γδ T Cells and Innate Lymphocytes Initiate Psoriasis-like Inflammation in Mice

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And partially on the following submitted manuscripts

Langerin^{neg} conventional DC-derived IL-23 drives psoriatic plaque formation in mice

Christian Wohn, Julia L. Ober-Blöbaum, Stefan Haak, Stanislav Pantelyushin, Cheolho Cheong, Sonja P. Zahner, Sabina Onderwater, Marius Kant, Heike Weighardt, Bernhard Holzmann, Boris Reizis, Burkhard Becher, Errol Prens and Björn E. Clausen

Psoriasis: γδ T cells and IL-17 go under the skin?

Burkhard Becher & Stanislav Pantelyushin

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Summary

Psoriasis is a common, relapsing inflammatory skin disease characterized by erythematous scaly plaques. It is thought to develop through a combination of genetic and environmental factors. Currently it is widely held that psoriasis is mediated by auto-aggressive helper T cells. Despite the availability of new effective drugs to treat psoriasis, the underlying mechanisms of its pathogenesis are still poorly understood. Recent studies have shown that Aldara cream, used to treat benign skin abnormalities, triggers psoriasis-like disease in humans and mice and have implicated T_H17 cells in the disease initiation. Using this as a model, we found a predominant role for the T_H17 signature cytokines IL-17A, IL-17F, and IL-22 in psoriasis-like plaque formation in mice. Using gene-targeted mice, it was observed that loss of *Il17a*, *Il17f*, or *Il22* strongly reduced disease the severity of psoriasis. However, we found that T_H17 cells were not the primary source of these pathogenic cytokines. Instead, IL-17A, IL-17F, and IL-22 were produced by a skin-invading population of γδ T cells and RORγt$^+$ innate lymphocytes. Furthermore, our findings establish that RORγt$^+$ innate lymphocytes and γδ T cells are necessary and sufficient for psoriatic plaque formation in an experimental disease model that closely resembles human psoriatic plaque formation. These findings together with the matching clinical observations inevitably lead to a paradigm shift in our understanding of the human disease pathogenesis implicating epithelial stress responses and innate immunity to contribute to or even dominate the initiation of psoriasis.

Zusammenfassung

Psoriasis ist eine häufige, rezidivierende entzündliche Hauterkrankung, die sich durch gerötete schuppige Plaques kennzeichnet und sich vermutlich durch eine Kombination von genetischen und umweltbedingten Faktoren entwickelt. Nach heutiger Lehrmeinung ist Psoriasis durch auto-aggressive T-Helfer-Zellen vermittelt. Trotz der Verfügbarkeit von neuen wirksamen Medikamenten zur Behandlung von Psoriasis, sind die zugrunde liegenden Mechanismen der Pathogenese noch weitgehend unverstanden. Jüngste Studien haben gezeigt, dass Aldara Creme – normalerweise eingesetzt zur Behandlung von gutartigen Hautveränderungen – eine Psoriasis-ähnliche Krankheit auslöst. Sowohl bei Menschen als auch bei Mäusen scheinen T_H17-Zellen zur Krankheitsentstehung beizutragen. In dieser experimentellen Psoriasis konnten wir eine herausragende Rolle für die T_H17 Zytokine IL-17A, IL-17F und IL-22 bei der Psoriasis-ähnlichen Plaquebildung bei Mäusen nachweisen. Bei Verwendung von transgenen Mäusen zeigte sich, dass der Verlust von IL-17A, IL-17F oder IL-22 die Schwere der Erkrankung stark reduziert. Jedoch scheinen T_H17 Zellen nicht die Hauptquelle dieser pathogenen Zytokine zu sein. Stattdessen wurden IL-17A, IL-17F, und IL-22 durch eine Population von γδ T-Zellen und RORγt angeborenen Lymphozyten produziert, die in die Haut einwandern. Außerdem scheinen RORγt positive angeborene Lymphozyten und γδ T-Zellen für die Plaque-Bildung in unserem Krankheitsmodell, welches die humane psoriatische Plaquebildung nachahmt, notwendig und ausreichend zu sein. Diese Ergebnisse, zusammen mit den passenden klinischen Beobachtungen, werden unweigerlich zu einem Paradigmenwechsel in unserem Verständnis der menschlichen Krankheitsentstehung führen: Epitheliale Stressreaktionen und angeborenen Immunität tragen dazu bei oder dominieren sogar die Entstehung der Psoriasis.

Abbreviations

APC	Antigen presenting cell
ATP	Adenosine 5´-triphosphate
CD	Cluster of differentiation
CFA	Complete Freud's adjuvant
CLA	Cutaneous leukocyte antigen
DC	Dendritic cell
dDC	Dermal dendritic cell
DETC	Dendritic epidermal T cell
GM-CSF	Granulocyte Monocyte-colony stimulating factor
GWAS	Genome-wide association studies
H&E	Hematoxylin & Eosin
HLA	Human leukocyte antigen
i.v.	intravenous
ICAM	intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
iNOS	Inducible nitic oxide synthase
LTi	Lymphoid Tissue inducer
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
NF-κB	Nuclear factor-κB
NK-cell	Natural killer cell
NKT-cell	Natural killer T-cell
PAMP	Pathogen associated molecular pattern
PASI	Psoriasis area severity index
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
RAG	Recombination activating gene
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
T_H	T-helper cell
T_{reg}	Regulatory T-cell
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor
WT	wild-type

1. Introduction

The immune system has evolved under selective pressure from the pathogens. This resulted in multicellular organisms developing diverse mechanisms to protect themselves by detecting the microorganism and subsequently neutralizing or killing them. These mechanisms are old and highly conserved and are termed the innate immune system. Its recognition receptors are encoded within the genomes of the species[1].

On the other hand, the adaptive immune system relies on receptors, which are generated during maturation of each organism. This mechanism leads to *de novo* generation of a diverse repertoire of receptors with random specificities. As a result, the recognition of these receptors is highly specific, but at the same time it is determined by chance. This is also the case for the subsequent response of the cell in the event of receptor engagement. Different pathogenic insults require different and appropriate responses. Hence, the adaptive immune system requires informative signals from the innate immune system to mount or not and the type of immune response required[2]. Moreover, an accidental event of recognition of self-antigens by these receptors could lead to autoimmunity.

Innate immune cells include dendritic cells (DCs), macrophages, and neutrophils, among others. The innate immune system is centered around recognition of pathogen-associated molecular patterns (PAMPs), rather than specific organisms or antigens[3]. The PAMPs on the pathogens are recognized by the receptors encoded and expressed by the innate immune cells and are called pattern recognition receptors (PRRs)[4]. The most well-studied class of PRRs are the Toll-like receptors (TLRs)[5]. Engagement of the PRRs increases the phagocytic activity of the innate immune cells, which subsequently leads to the destruction of pathogens. It is thought that the innate immune system determines the origin of antigens through differential receptor signals. Subsequently, it coordinates the adaptive immune response by means of antigen presentation, costimulation as well as through production of cytokines and chemokines. The latter signals control the recruitment of leukocytes to the sites of insult and regulate the activation of appropriate effector mechanisms, for example by controlling differentiation of T lymphocytes into effector cells of a particular type[6].

The T and B lymphocytes are the two principle cell types that make up the adaptive

immune system. Only these cells express highly variable, randomly generated, clonally distributed antigen receptors and need antigen priming for effective antigen responses. B cells provide antibody responses, and T cells are the source of cell-mediated immunity. The adaptive immune system evolved to "remember" pathogenic insults, making the immune response more efficient and rapid.

The generation of B cells occurs throughout life and takes place in the bone marrow. According to the clonal-selection hypothesis of Frank Macfarlane Burnet, recognition of foreign antigen by mature B cells should trigger clonal expansion and antibody secretion, thereby yielding immune specificity and memory. At the same time, reactivity to autoantigens early in development should trigger cell death, thereby ensuring tolerance to self[7]. B-cell tolerance is achieved by clonal deletion, cell inactivation (anergy) or receptor editing. Receptor editing is the dominant tolerance mechanism for developing B cells, and occurs during the immature B-cell stage, in the bone marrow[8].

At the same time, thymic selection processes shape the T-cell repertoire. During positive selection thymocytes that do not engage self-MHC molecules die by neglect. Later, negative selection eliminates thymocytes that have high-avidity interactions with APCs presenting a self-antigen, by clonal deletion[9]. Moreover, medium self-reactive thymocytes can undergo anergy, T-cell receptor revision, through editing or be diverted to other lineages (T_{reg} or CD8αα). Finally, thymocytes that have a TCR with low affinity for self-peptide–MHC complexes are positively selected to further differentiate and function in adaptive immunity, and this process is also known as central tolerance[10].

The large numbers of all body proteins are not expressed in the thymus or in the serum. The process of preventing self-reactivity after the lymphocytes leave the thymus/bone marrow is called peripheral tolerance. Autoreactive T and B-lymphocytes may ignore the presence of their autoantigen due to low concentration of the antigen or immunoprivileged location of its expression. T cells are only able to recognize processed antigens presented in the context of MHC molecules[11]. Peripheral anergy results from naïve T cells encountering their antigen-MHC complex, but without co-stimulation. Autoreactive lymphocytes can also be eliminated by lymph node–resident immature dendritic cells that present tissue-derived antigen. Finally, autoagressive T lymphocytes can be suppressed by regulatory T cells[12]. Despite all of these sophisticated and clever tolerance

mechanisms unwanted inflammation can still occur. The milder version results in allergy, but is, strictly speaking, not an autoimmune process[13]. Autoimmunity on the other hand can result in severe inflammation and currently seems to be on a rise[14].

The skin together with the mucosal surfaces act as important barriers to protect the body from the outside environment and microbial threats. To maintain the barrier integrity tight regulation and clockwork homeostasis are required to keep the skin barrier intact. However, when the barriers are breached, for example tissue injury, a rapid, but at the same time self-limiting immune response is required. Inadequate responses can lead to severe infections and tumourogenesis[15], whereas excessive responses can result in chronic inflammation and autoimmunity.

Psoriasis is a common skin and joint auto-inflammatory disease. It occurs from a complex combination and interplay of genetic predisposition and environmental factors that lead to excessive immune responses to a self or environmental antigen, resulting in chronic inflammation[16,17].

1.1 Clinical features and hallmarks of psoriasis

Psoriasis was originally thought to be variant of leprosy[18] and it took until 1841 for it to be classified as a separate disease[19,20]. It is a chronic relapsing and remitting skin and joint disease that affects approximately 2-3% of the world's population[16,21].Psoriasis has a bimodal distribution with peaks between 15 and 30 years, as well as 50 and 60 years[22].

1.1.1 Epidemiology

The highest prevalence of psoriasis is observed in Caucasian North Americans, affecting nearly 5% of the population. At the same time only about 0.5% of Africans and Asians are affected[16,21]. A population study suggests that psoriasis has a negative effect on overall longevity even after all the common mortality factors are accounted for. This results in an overall decrease in life expectancy of 3.5 years in males and 4.4 years in females[20,23]. The high morbidity in psoriasis patients results in its burden on the economy being similar to that of diabetes, cardiovascular diseases and CNS illnesses[24-26]. Increases in suicidal tendencies, depression and mental illnesses have also been attributed to psoriasis[27]. Moreover, income and employment are negatively impacted among patients with severe psoriasis compared with mild psoriasis

patients[28].

Psoriasis is generally limited to inflammation of the skin, but as many as 30% of the patients also suffer from debilitating psoriatic arthritis. At the same time psoriatic arthritis prevalence in the general population is around 1%[29,30].

Psoriasis has many well-established comorbidities, in particular cardiovascular diseases[31,32]. Severe psoriasis has been shown to be an independent risk factor for myocardial infarction in younger patients[20,33]. Other diseases associated with psoriasis are: Crohn's disease[34], type II diabetes[35], obesity[36-38], metabolic syndrome[39] and lymphoma[40].

1.1.2 Clinical features

Psoriasis patients can show a wide variety of clinical phenotypes that represent a dynamic spectrum of a single disease (Figure 1)[16,19,20]. Most scientific research refers to the most common form of the disease called psoriasis vulgaris (Figures 1A, B, D) and accounts for nearly 90% of the cases[21]. The disease is generally manifested as raised, well-demarcated, erythematous oval plaques with adherent silvery scales[20,41].

The acute form of the disease, during initial eruptions may exhibit a guttate distribution pattern and is often triggered by streptococcal infections (Figure 1E)[42,43].

Inverse psoriasis is usually located at intertriginous areas and rarely displays any scaling (Figure 1C). Other versions such as generalized pustular psoriasis of von Zumbusch may also occur (Figure 1F)[44].

The classification of the disease has been historically based on the clinical appearance and mainly on plaque thickness[45]. Alternatively, psoriasis is classified according to HLA status, which determines type I and type II psoriasis[46,47]. Type I psoriasis accounts for about 65% of the cases and generally occurs in younger fraction of the bimodal distribution, with a positive family history of the disease, preceded by streptococcal throat infection and guttate lesions. Type II psoriasis is associated with the older patients, with no family history of the disease. It is generally not linked to any preceding disease and tends to lead to involvement of nails and joints (Figure 1G)[20,48].

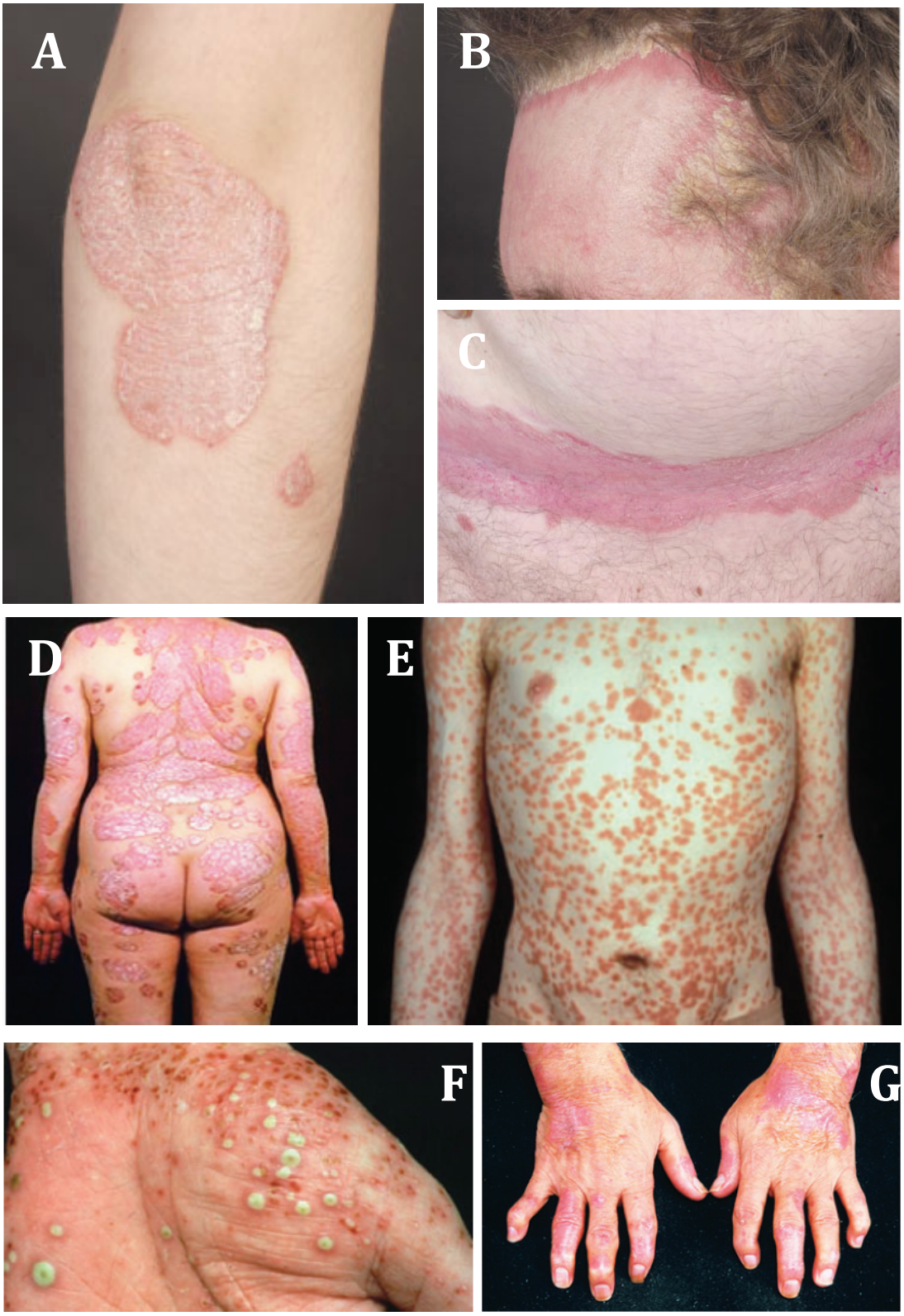

Figure 1. Clinical images showing the spectrum of psoriasis phenotypes. The typical psoriatic lesion is a sharply demarcated erythematous plaque covered by silvery white scales, (A) often appearing on the elbows. (B) Scalp involvement is seen in approximately 50 percent of patients with psoriasis. (C) Inverse psoriasis (D) Lesions may cover the entirety of the body (E) Guttate psoriasis is often triggered by streptococcal infections. (F) Pustular psoriasis. (G) Psoriatic arthritis (Adapted from Perera et.al., 2012; Nestle et. al., 2009, and Schon and Boehncke 2005)

1.1.3 Histological features

The thickening of the epidermis and the associated scales are due to premature keratinocyte maturation and subsequent incomplete cornification, leading to the retention of the nuclei in the stratum corneum (parakeratosis). Abnormal increase in the keratinocyte turnover causes thickening of the epidermis (acanthosis) and elongation of the epidermal rete ridges (papillomatosis), as well as loss of the granular layer (hypogranulosis)[20,41]. There is extensive new blood vessel formation[49], which results in pinpoint bleeding upon damage to the scales. Finally, a mixed leukocytic infiltrate is seen in both dermis and epidermis, composed of DCs, $CD4^+$ T_H cells within the upper papillary dermis and $CD8^+$ cytotoxic T cells within the epidermis[50]. The neutrophilic granulocytes transmigrate through the epidermis and form, a histopathological hallmark of psoriatic lesions, the Munro microabscesses[19,51] (Figure 2).

1.2 Genetics

The population and family studies that have started in 1960s showed that psoriasis occurs more often in relatives than within controls and general population[52,53]. The studies in twins have shown genetic predisposition to psoriasis as concordance in monozygotic twins was 35-70%, while only 12-20% in dizygotic twins[54-56]. No clear inheritance pattern and lack of 100% concordance in twins hints at a role of environmental factors in psoriasis pathogenesis[57,58]. Over the last half-century many advances have been made in understanding the genetic basis of psoriasis, identifying several predisposition loci and genes. These were identified using genome-wide scale and candidate-gene as well as genome-wide association studies (GWAS).

1.2.1 Psoriasis susceptibility (PSORS)

The major genome region associated with psoriasis is called psoriasis susceptibility 1 (PSORS1). It is a 220 kb fragment, encoding the genes of the major histocompatibility complex (MHC), located on the short arm of chromosome 6 and contains 10 known genes[59]. This region was first identified as potential psoriasis locus, when serologic typing identified an association between psoriasis and the *HLA-Cw*602* allele of the MHC class I molecule human leukocyte antigen molecule HLA-C[60,61]. Sequence, haplotype analyses[62] and fine linkage mapping have linked HLA-C

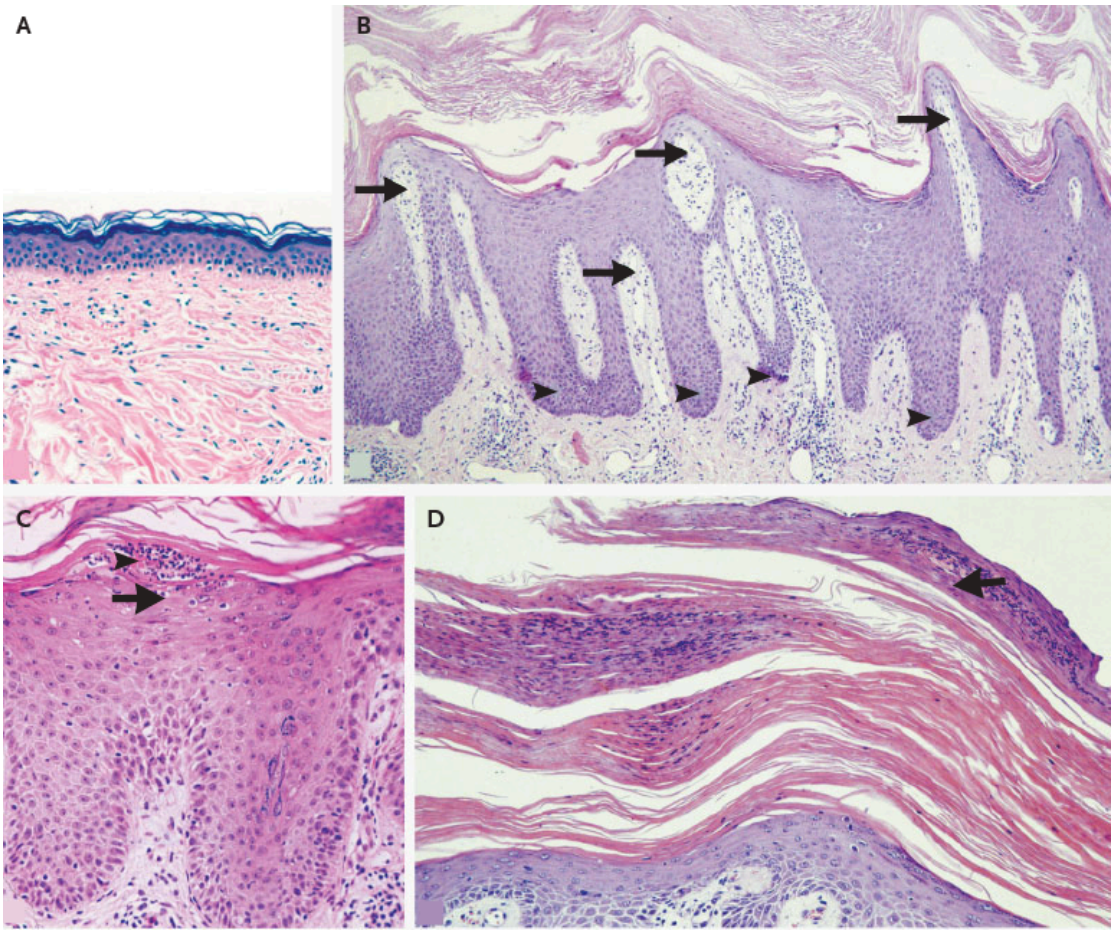

Figure 2. Hematoxylin and eosin stainings showing histological features of psoriasis. (A) Normal skin. (B) Psoriatic skin showing acanthosis, elongation of epidermal rete ridges (arrowheads), marked hyperkeratosis, loss of the granular layer, and parakeratosis. Dermal blood vessels are increased in number and size. They are contorted and reach up to locations directly underneath the epidermis (arrows). (C) A mixed leukocytic infiltrate is seen in both dermis and epidermis. Neutrophilic granulocytes transmigrate through the epidermis and form Munro microabscesses underneath the stratum corneum (arrowhead). (D) As the lesions progress, these microabscesses are transported to the upper layers of the stratum corneum, where they slough off (arrow). Adapted from Schon and Boehncke 2005.

variant *HLA-Cw*060* to be the most probable PSORS1 gene. Using single nucleotide polymorphism (SNP) studies within the HLA-C gene it was later shown to have the greatest association with psoriasis in multiple GWAS[63,64]. However, at the same time these studies hinted at other potential determinants of psoriasis susceptibility within the MHC[20].

As many as 60% of psoriasis sufferers carry the *HLA-Cw*602* gene-variant, which confers a stunning 20-fold-increased risk of developing psoriasis and is found in 10-15% of the population[65]. Moreover, individuals homozygous for this gene variant are 5 times more likely to develop the disease compared to heterozygous ones[66]. The

patients with the *HLA-Cw*602* allele are part of psoriasis type I group described above[47,48].

The exact role of *HLA-Cw*602* and HLA-C in psoriasis is still unknown. This is due to high homology MHC class I genes and high degree of polymorphisms within HLA-C, which impede functional studies[20]. As HLA-C is expressed on antigen presenting cells (APCs) it has the ability to influence both innate and adaptive immune systems. Through these molecules APCs can prime cytotoxic $CD8^+$ T cells, which are commonly found in the psoriatic epidermis[50]. Moreover, keratinocytes that express HLA-C can interact with killer immunoglobulin-like receptors (KIRs). These molecules are expressed on NK and NKT cells and have both activating and inhibitory functions[67]. Interestingly, the latter cell type have recently been reported to play a role in psoriasis[68]. It is also possible that unique features of *HLA-Cw*602* that affect its expression and/or activity can lead to modified innate and adaptive immune responses that eventually lead to psoriasis[20].

Even though PSORS1 confers the highest risk for the development of psoriasis, it is responsible for only about 50% of familial clustering. Other studies using linkage analyses of families with multiple psoriasis sufferers have identified another 9 psoriasis susceptibility loci (PSORS2-10)[20,46].

Another region of strong association with psoriasis, PSORS2 is associated with chromosome 17q25 and is separated by 6 Mbp. The associated SNPs in the proximal peak lie in or near *NAT9* gene, a member of the N-acetyltransferase family, and *SLC9A3R1*. SLC9A3R1 is implicated in diverse aspects of epithelial membrane biology and immune synapse formation in T cells. The distal peak of association is in *RAPTOR,* a target of rapamycin (TOR)-scaffold protein. Expression of SLC9A3R1 is highest in the uppermost stratum Malpighi of psoriatic and normal skin and in inactive versus active T cells. Another disease-associated SNP lies between *SLC9A3R1* and *NAT9*, and leads to loss of RUNX1 transcription factor binding[69-71].

Psoriatic skin lesions demonstrate an activated IFN-α signaling pathway[72]. Continuous excessive IFN-α signaling in IFN regulatory factor deficient (*IRF-2-/-*) mice causes an inflammatory skin disease resembling psoriasis[73]. At the same time, treatment of psoriasis patients with recombinant IFN-α for unrelated conditions can exacerbate psoriasis[74,75]. Finally, in the xenotransplantation model of psoriasis blocking of IFN-α resulted in complete disease prevention[76]. Eventually all this evidence resulted in PSORS3 being mapped to the *IRF2* gene on chromosome 4p, a

transcriptional repressor of type I interferon target genes[77,78].

Cystatin A is involved in regulation of the skin barrier. It is now believed to be one of the precursors of the cornified cell envelope formed during terminal differentiation of keratinocytes, thereby suggesting that cystatin A expression is associated with keratinocyte differentiation, which is impaired in psoriatic skin. Recently, *cystatin A* gene has been mapped to PSORS5 locus on chromosome 3q[79,80].

PSORS6, 8 and 9 loci are not as well characterized, but they have implicated *JUNB*[81,82]; *CXCL1*, *CX3R1*, *CARD15*[71]; and *Il15*[83] genes, respectively, in the pathogenesis of psoriasis.

1.2.2 IL-23/T_H17 Pathway genes

Many studies have shown a crucial role for IL-23 and T_H17 cells in the pathogenesis of psoriasis[84-86]. IL-23 is a heterodimeric IL-12 family cytokine[87]. It consists of a unique IL23p19 subunit and IL12/23p40 subunit, shared with IL-12. Similarly, IL-23 signals through a heterodimeric receptor composed of IL-23R and IL-12Rβ1 (Figure 3). Receptor binding by IL-23 leads to a signaling cascade involving signal transducer and activator of transcription 3 (STAT-3)[87].

IL-23 plays a crucial role in T_H17 cell differentiation, as well as induction of IL-17 and IL-22 in other cell types[88]. Furthermore, this pathway has been identified to carry the major pathogenic functions in many autoimmune diseases[89].

Multiple studies have identified SNPs associated with psoriasis within *IL12B* and *IL23R* genes, which code for IL-12/23p40 and IL-23R, respectively[90,91]. Notably, *IL23R* actually lies within PSORS7 locus[92]. Disease associated SNPs within IL-23p19 subunit gene, *IL23A* have also been identified[64].

One of the most well described polymorphisms identified by GWAS is guanine-to-adenine missense SNP within *IL23R*, which results in Arg381-to-Gln within the cytoplasmic domain of IL-23R and is associated with protection against psoriasis[64,90,91]. The same substitution is also associated with protection from Crohn's disease[93] and ankylosing spondylitis[94].

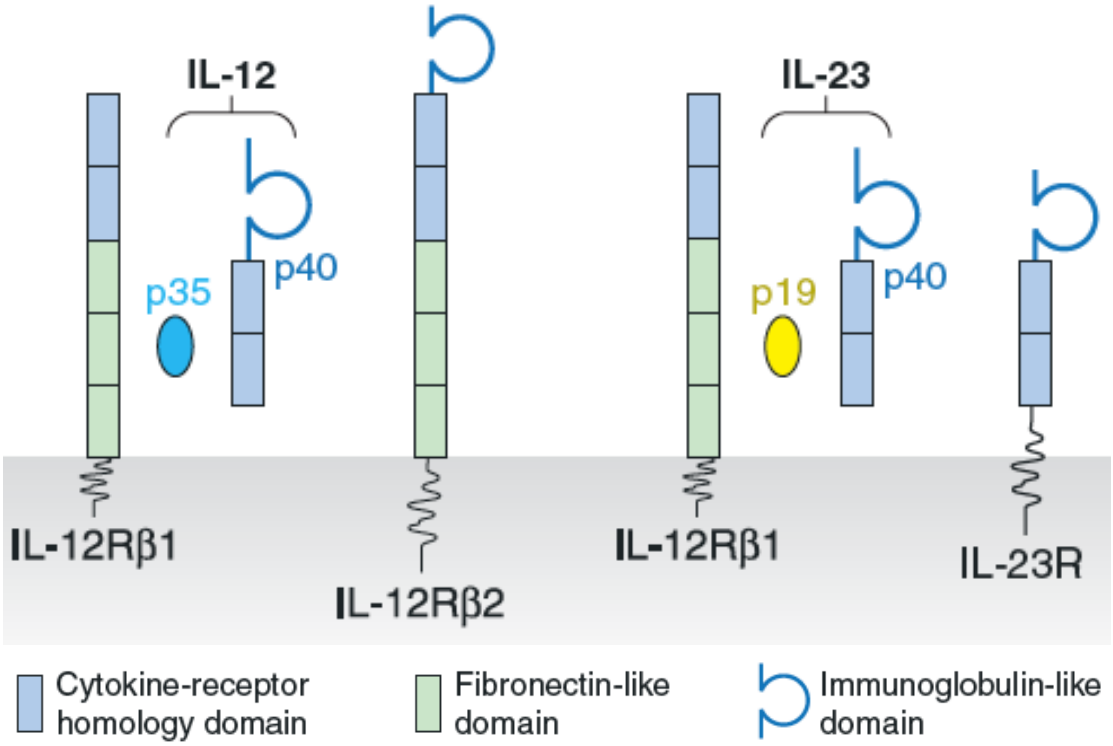

Figure 3. Structures of IL-12, IL-23 and their receptors.

Modified from Kastelein et.al., 2007

The more rare *IL23R 381Gln* gene variant accounts for threefold protection against Crohn's disease[95] and twofold protection against psoriasis[96] and ankylosing spondylitis[94]. These findings also imply that this SNP may offer protection for many other autoimmune diseases, in which the IL-23/T_H17 pathway is involved[89,95]. Functional studies of this substitution have shown that, despite it having no effects on T_H17 cell numbers and activity, the effector functions of T_H17 cells were lower in the protective allele carriers. STAT-3 activation and IL-17A secretion were reduced in response to IL-23 in the cells derived from the donors heterozygous for the protective allele[97]. Moreover, these findings are being used as a platform to investigate the possibility of using *IL23R 381Gln* variant as a biomarker to predict therapeutic responses[20].

1.2.3 NF-κB pathway genes

The NF-κB pathway is critical for immune responses and cell cycle regulation. The genes of this pathway encode for proinflammatory, cell cycle, antiapoptotic and

chronic inflammation mediators[98,99]. In resting cells NF-κB proteins are localized in the cytoplasm and associate with inhibitory proteins of this signaling pathway, such as inhibitor of κB (IκB). This pathway can be triggered by a multitude of signals, including TNFα, IL-1, TLR signals, and free radicals. Upon activation of the signaling cascade IKK activation results in IκB phosphorylation and degradation, for the canonical pathway, or p100 processing to p52 for the noncanonical pathway. Phosphorylated NF-κB dimers bind to κB DNA elements and induce transcription of target genes[99].

Activation of NF-κB may play an important role in psoriasis, as multiple GWAS studies, have identified numerous SNPs within the genes coding for NF-κB signaling regulators. These include downstream TNF signaling genes *TNFAIP3*[64,100] and *TNIP1*[64,100,101]. *NFKBIA*, which codes for α subunit of IκB[100,102] and *TRAF3IP2*[100,103,104], coding for adaptor molecule involved in IL-17 activation of NF-κB signaling have also implicated the NF-κB pathway in psoriasis. T_H17 and NF-κB are the two major proinflammatory pathways involved in psoriasis, and *TRAF3IP2* may represent a critical checkpoint regulating both the adaptive and innate immune responses that underpin psoriasis pathogenesis[20].

1.2.4 Epidermal differentiation genes

PSORS4 accounts for a cluster of about 20 genes that are expressed during epidermal differentiation[105,106], confirmed both by GWAS[107] and genome-wide copy number variation analysis[108]. Of particular importance are three genes of the late cornified envelope (LCE) family. Interestingly, SNPs in the LCE cluster are associated with psoriasis in Chinese psoriasis patients[107], while deletions of regions encoding for LCE3C and LCE3B are common in European psoriasis patients[108]. Moreover, LCE3C mRNA is significantly upregulated in wounded and psoriatic skin, indicating its importance in skin barrier repair[20].

1.2.5 Other genes

Another important gene implicated in psoriasis susceptibility and replicated in multiple studies is *ERAP1*[100,101]. ERAP1 is an IFN-γ-induced aminopeptidase, involved in trimming peptide antigens for binding to MHC class I[109]. Accordingly, it influences psoriasis susceptibility only in individuals carrying *HLA-C* risk-associated

variant[100].

1.3 Current understanding of the pathogenesis of psoriasis

Despite the exact triggers of psoriasis still unknown there are many associated triggers: streptococcal throat infection[43,110]; physical trauma, such as tattoos and scratches, also known as Koebner phenomenon[111]; some medications, including antidepressants, antihypertensives, and anticytokine treatments for other conditions[112]; smoking, obesity and alcohol[20].

The current understanding of the pathogenesis of psoriasis implicates complex interplay between disruption of the skin barrier and deregulated immune compartment, which results in extended inflammatory response[20]. Moreover, there are many parallels between skin healing and psoriasis, which leads many to believe that psoriatic lesions represent a prolonged wound-healing process[113-115].

Currently, there is still an ongoing argument about the dominant cell type in psoriasis pathogenesis, with keratinocytes and T cells as primary suspects. At the same time if innate or adaptive immune responses are more important for the disease development[20]. Complicating it even further is the dynamic nature of psoriasis, which makes it likely that different cell types play different roles during initiation, progression, maintenance and remission of the disease[46]. In addition, despite general agreement about a role of T cells in psoriasis, no foreign antigens or autoantigens have been identified and yet autoimmune nature of psoriasis has long been postulated[116]. Only very recently this theory has received a boost, when self-DNA and self-RNA were found to induce proinflammatory cytokine signals[117].

Cell types that have been identified within psoriatic lesions include mDCs, pDCs, macrophages, neutrophils and T cells, with apparent increased vascularisation[118-120]. In the presence of an altered and deregulated keratinocyte barrier, these cells continue to orchestrate an aberrant immune response to an unknown antigen, resulting in the development of psoriasis[20].

1.3.1 Keratinocytes

Keratinocytes are the main constituents of the epidermis. In addition, to their structural and mechanical barrier functions, they have an important role in regulation

of the inflammation in the skin[121]. Similar to the innate immune system they respond to pathogenic insults in a rapid nonspecific manner. Despite not being professional APCs keratinocytes are able to process and present antigens to T cells. There is also close association between Langerhans cells, T cells and keratinocytes in the skin[122,123].

The major factor determining the immune activity of keratinocytes in normal or diseased skin is their state of activation or differentiation. Terminally differentiated keratinocytes are important for mechanical integrity of the skin barrier as well as protection against pathogens and immune responses against those[124]. Under normal conditions, differentiation is favoured and only basal keratinocytes can regenerate and differentiate through the spinous and granular layers of the epidermis to become corneocytes[20].

In psoriasis, terminal differentiation of keratinocytes is not complete and keratinocyte stem cell proliferation is deregulated, resulting in preferential activation and proliferation of cells that mature too quickly, but differentiate incompletely[120]. This leads to increased responses to cytokines and growth factors, which is also observed in wound healing. Activated keratinocytes display different phenotype compared to terminally differentiated ones, they are hyperproliferative and display migratory features. Additionally, they can rearrange their cytoskeleton, increase the expression levels of cell surface receptors and secrete components of the basement membrane. Moreover, activated keratinocytes are able to produce cytokines, proangiogenic molecules, such as VEGF, which help to restore tissue integrity and recruit of circulating leukocytes[49,125]. Upon successful repair of the skin barrier keratinocytes are deactivated and return to their differentiated state, which is not the case in psoriasis[126].

1.3.1.1 IL-1 Family members and the inflammasome

The critical factor for keratinocyte activation is IL-1, which is important for many inflammatory responses to injury[127]. Isoforms of IL-1, termed α and β, are stored in the cytoplasm in their inactive pro-forms, under normal conditions[128-131]. When PRRs are triggered, caspase-1 is activated within the inflammasome of keratinocytes[132], resulting in cleavage of IL-1 isoforms, as well as pro-IL-18 into their active forms[133]. Injured or activated keratinocytes release active IL-1, thus allowing the neighboring

skin cells to respond to the insult by amplifying the response through further secretion of IL-1, TNFα, IL-6 and other proinflammatory mediators[134-136].

Apart from having proinflammatory effects on surrounding cells IL-1 also acts as a chemoattractant for lymphocytes, enabling them to extravasate and migrate to the injured areas[137]. This is additionally mediated through induction of selectin expression on endothelial cells, allowing lymphocytes to slow down, firmly attach and subsequently enter the damaged tissues. Moreover, IL-1 has further effects on T cells, by skewing them towards IL-17 production[138,139]. At the same time another member of this family, IL-18 promotes polarization towards T_H1 phenotype[140]. A microarray study showed that many members of IL-1 family, including IL-1F6, IL-1F8 and IL-1F9 are upregulated in psoriatic skin[141,142].

In psoriatic lesions keratinocytes also produce other immune mediators such as intercellular adhesion molecule 1 (ICAM-1), CD40, HLA-DR and S100 family proteins, which act as chemotactic factors for leukocytes[143]. At the same time there is a positive feedback loop resulting in infiltrating leukocytes further upregulating the above molecules[46,118,123]. Additionally, the leukocytes that have already entered the skin and keratinocytes produce chemokines, resulting in even more recruitment of the immune cells into the skin. Most notably, these are CCL27, ligand for CCR10, which is expressed by 90% of skin-infiltrating lymphocytes[144], as well as CCL4, CCL20, CXCL2, and IL-8[145]. Furthermore, keratinocytes can activate T cells directly through their expression of MHCII, ICAM-1 and B7-H1[146].

1.3.1.2 Antimicrobial peptides

Another very important component of keratinocyte immune responses are antimicrobial peptides, which have been shown to be highly upregulated in psoriatic skin[147]. There are multiple families of these peptides: cationic cathelicidins, defensins, S100 proteins, peptidoglycan recognition proteins, C-type lectins and iron-metabolizing proteins[148]. Antimicrobial peptides can also act as immune mediators[149], opsonins and chemokines[150,151]. IL-1 and IL-18 are both strong inducers of antimicrobial peptides[152,153]. One of these, LL-37 synergizes with IL-1 to induce even more cytokine and chemokine production, subsequently resulting in increased recruitment of neutrophils and macrophages, leading to even more inflammation[47,48,154].

1.3.1.3 Angiogenesis

Psoriatic skin is highly vascularized and development of high endothelial venules, usually associated with lymph nodes, is observed[20]. The blood vessels in the lesional skin are dilated and are highly permeable due to nitric oxide, produced by inflammatory DCs[155]. Higher vascularization is able to support more keratinocytes and also results in more leukocytes being recruited to the skin through higher levels of ICAM-1, vascular adhesion molecule 1 (VCAM-1) and E-selectin[125].

At the same time VEGF maintains endothelial cell integrity and induces their migration, survival, proliferation and its levels are increased in the sera of psoriasis patients, compared to healthy controls[156]. It's role in psoriasis is further supported through successes of antiangiogenic drugs such as AE-491 and paclitaxel[157,158].

1.3.2 Cells of the immune system

Many immune cells are associated with the psoriatic lesions, but as with many immune responses APCs are thought to respond to the initial trigger. These cells include including pDCs, mDCs, Langerhans cells and dermal resident DCs (dDCs). On the other hand the key effector cells activated by DCs are T cells, which through production of proinflammatory cytokines: TNF-α, IFN-γ, IL-17A, IL-17F, and IL-22 induce the skin alterations that are observed in psoriasis[20].

1.3.2.1 Dendritic cells

DCs are the sentinels of the innate immune system that bridge the innate and the adaptive immune systems. These cells are called professional antigen presenting cells that prime and polarize T cells to carry out antigen specific responses[159]. Langerhans cells and dDCs reside in the skin and therefore are well located to initiate inflammation in the skin in psoriasis[123]. At the same time pDCs are also thought to be highly involved in the psoriatic plaque formation[76,160].

1.3.2.1.1 Langerhans cells

Langerhans cells are epidermal resident specialized DCs, which were the first subset of DCs found to express langerin (CD207)[161]. Under normal conditions they are located within the basal and suprabasal regions of the epidermis in the immature state

and are closely associated with keratinocytes through their expression of E-cadherin[162]. Despite their epidermal localization the role of these cells in psoriasis still remains unclear[20].

1.3.2.1.2 Dermal dendritic cells

dDCs are much less studied than Langerhans cells due to the difficulty of isolating these cells. In mice, dDCs contain at least one additional population that, similarly to Langerhans cells, express langerin[163]. The classical langerin$^-$ dDCs comprise the majority of the dermal DC pool and express integrin CD11b. The recently identified langerin$^+$ dDC population represents 20% of the total dermal DC pool. Unlike Langerhans cells, langerin$^+$ dDCs express the integrin CD103[164]. For simplicity, the two dermal-resident DC populations are called CD103$^+$ DCs and CD11b$^+$ DCs.

Activated dDCs orchestrate the immune responses in the skin by secreting cytokines and chemokines to generate a cytokine milieu to effectively combat the insult. Most of the time this is beneficial and promotes the eradication of pathogens, but in rare cases it underlies a pathological tissue response with persistent inflammation. Activated dDCs produce both TNFα and inducible nitric oxide synthase (iNOS) are known as TIP (TNFα and iNOS-producing) DCs[161,165]. This type of DC has been proposed to have a major role in psoriasis through production of NO, resulting in vasodilation[165,166].

1.3.2.1.3 Plasmacytoid dendritic cells

pDCs are a rare population of DCs that are most well-known for their specialized anti-viral immune responses through production of type I IFNs[160,167]. They express high levels of TLR7 and TLR9 within their endosomes. The former recognizes single-stranded RNA and small-molecule immune response modifiers such as Imiquimod, resiquimod and gardiquimod (imidazoquinolines)[168,169]. There have been many clinical observations of 5% Imiquimod cream Aldara™ (3M) exacerbating or causing relapses of psoriasis[170-174]. Elevated levels of type I IFNs[72,175] as well as pDC numbers have also been reported in psoriasis[170,176]. These cells are not normally observed in the healthy skin, but have been found in both lesional and non-lesional psoriatic skin[76]. It is believed that pDCs are recruited to the skin by a fibroblast-derived molecule called chimerin. This molecule is abundantly expressed in prepsoriatic skin, adjacent to, and

within psoriatic plaques and is thought to promote the migration of pDCs from high endothelial venules to the skin[177]. In the xenotransplantation mouse model of psoriasis it was established that IFN-α produced by pDCs promoted activation and expansion of pathogenic T cells, leading to the development of psoriatic lesions. At the same time blocking IFN-α prevented T cell activation and lesion formation[76].

Under normal conditions pDCs are tolerant to self-DNA and -RNA, which arise from stressed or dying cells. However, it was shown that when these nucleic acids are bound to cathelicidin LL-37, they can trigger pDC activation through TLR7[117,178]. This event is thought to initiate the pathogenic cascade in psoriasis, allowing cross-talk between stressed or dying keratinocytes and pDCs and through the action of type I IFNs promotes maturation and activation of dDCs and mDCs[123].

1.3.2.1.4 Myeloid dendritic cells

Large numbers of mDCs have been found in psoriatic lesions, implying them in the disease pathogenesis[116]. Initial studies have shown that mDCs from psoriatic lesions mediate T cells responses, resulting in high levels of IL-2 and IFN-γ production. This lead to the initial belief that psoriasis is a T_H1-type disease[41,116,179,180].

The numbers of mDC in psoriatic dermis compared to uninvolved skin have been reported to be increased up to 30 fold[181]. In psoriasis, mDCs are believed to be activated by proinflammatory cytokines secreted by pDCs, such as type I IFNs and IL-6, as well as TNFα produced by keratinocytes[20]. More recently, it was also postulated that mDCs can be directly activated by LL-37-self-RNA complexes through TLR8, leading to amplification of TNFα and IL-6 production[178].

There is yet no clear understanding and differentiation between dDCs and mDCs in psoriatic skin. But it is thought that mDCs also contribute to psoriasis through IL-20[182] and IL-23 production[181,183]. TNF induces ICAM-1 expression by keratinocytes as well as other proinflammatory cytokines, such as IL-6 and IL-1 and chemokines: IL-8, CCL20[20]. IL-20 can directly act on keratinocytes and induce their proliferation[184]. Moreover, with the recent shift in the understanding of the psoriasis pathogenesis, IL-23, for which DCs are the main source, induces polarization of T cells towards a T_H17 phenotype[183,185,186]. Finally, it was recently suggested that fibroblasts can augment production of IL-23 by DCs through prostaglandin 2[187].

1.3.2.2 Macrophages

Macrophages can also act as APCs, even though they are not as efficient as DCs[188], they are thought to be involved in psoriasis through TNF production[189]. Some macrophages also reside in the dermis and under inflammatory conditions migrate to the lymph nodes[190]. Additionally, their role in psoriasis may be due to their abilities to efficiently phagocytose tattoo particles and to regulate angiogenesis[191,192].

1.3.2.3 T cells

The initial response to the stimulus results in the release of cytokines, chemokines and growth factors by keratinocytes and DCs. Following this it is believed that activated lymphocytes, which have been primed by the DCs in the lymph nodes and have upregulated skin homing receptors, extravasate into the dermis through increased vascularization and adhesion molecule upregulation. Under normal conditions more $CD4^+$ T_H cells reside in the dermis than in circulation[193]. At the same time epidermal lymphocytes are very rarely observed and the majority of these are $CD8^+$ T cells[123].

In the lesional psoriatic skin activated memory T cells are primarily $CD4^+$ T_H cells in the dermis, and $CD8^+$ T cells in the epidermis[145,179,194]. These findings and successful treatment of psoriasis patients with anti-T cell therapies have shifted the paradigm of keratinocytes being the main cellular players in psoriasis in favour of T cells[195-199]. These findings were further supported by the findings during allogeneic bone marrow transplantation[200,201], indicating that psoriasis can be transferred through hematopoietic cells. Further reinforcement of this theory was provided by the studies in the mouse models of psoriasis (discussed later)[202-205].

Until recently, only T_H1 and T_H2 phenotypes of T helper cells were known. Psoriasis was considered to be a T_H1 disease, while atopic dermatitis was classified as T_H2 disease[206,207]. However, with the recent discovery of so-called T_H17 cells[17,20,208,209] and other differentially skewed phenotypes of T helper cells[210-213] this belief initially turned into a duality (T_H1/T_H17)[16,84,85] and even more recently into a triumvirate ($T_H1/T_H17/T_H22$)[86] (Figure 4)[20]. There are variable beliefs about T helper cell flexibility[214] within the psoriasis and immunological field in general[16,20,215]. Therefore, it still remains to be determined, which cytokine producing profile is more important or if differential polarization of T helper cells occurs during different stages of the disease.

1.3.2.3.1 Regulatory T cells

T_{reg} cells are specialized T helper cell subset that represents 1-5% of peripheral $CD4^+$ T cells. They are important for suppression of self-reactive T cells, self-tolerance and are characterized by expression of the transcription factor forkhead box protein 3 (Foxp3), as well as high levels of CD25 and CTLA-4[216-218]. Their exact role in psoriasis is yet to be fully elucidated[219] as in some studies these cells seem to be dysfunctional[220] and in others they do not seem to be affected in psoriasis[221].

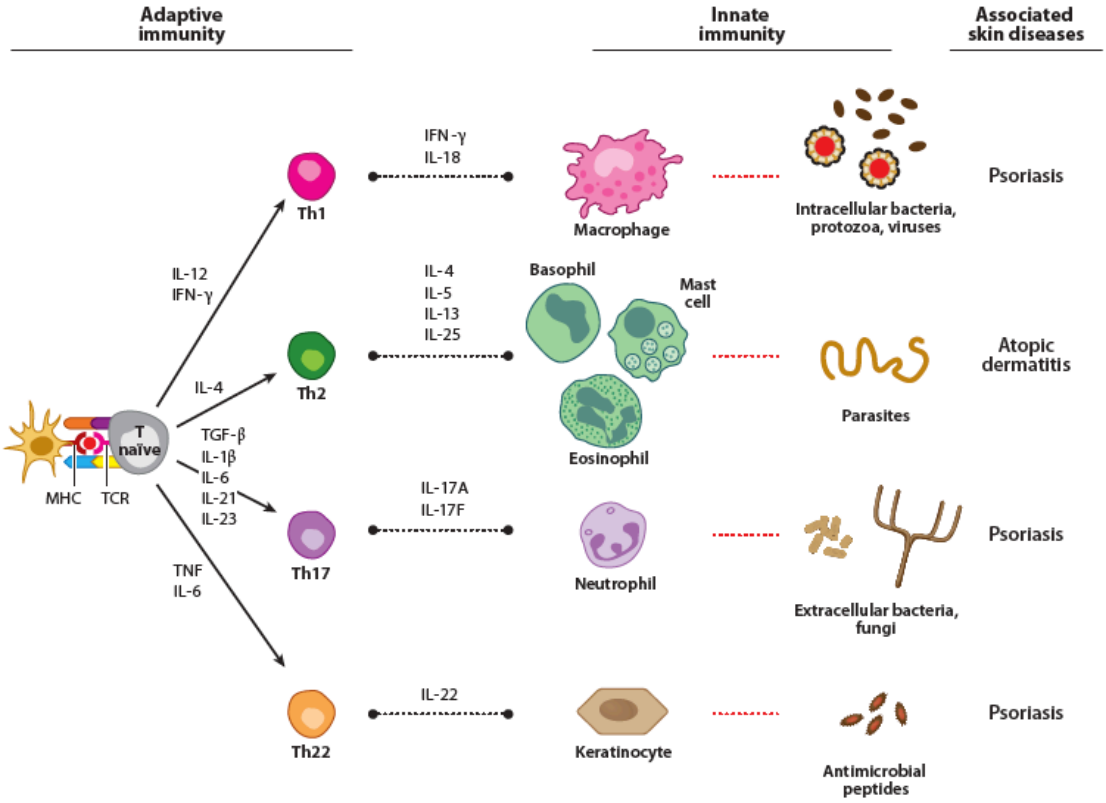

Figure 4. Effector subsets of T helper cells and their roles in psoriasis. $T_H1$cells differentiate in the presence of IL-12, IL-18 and IFN-γand produce IFN-γ. This, in turn, facilitates macrophage-mediated immunity against intracellular bacteria, protozoa, and viruses. T_H2 cells develop in the presence of IL-4 and release IL-4, IL-5, IL-13, and IL-25. T_H2 cells are important for cellular immunity against parasites and helminths mediated by basophils, eosinophils, and mast cells, as well as components of humoral immunity. T_H17 cells require a combination of TGF-β and proinflammatory cytokines (IL-1β, IL-6, IL-21, and IL-23) to differentiate. They produce IL-17A, IL-17F, and IL-22 and are important in neutrophil-mediated protection against extracellular bacteria and fungi as well as in keratinocyte production of antimicrobial peptides. Recently identified T_H22 cells differentiate in the presence of TNF and IL-6 and produce IL-22. IL-22 acts on epithelial cells, for instance, keratinocytes, which proliferate and increase their production of antimicrobial peptides. Adapted from Perera et.al.,2012.

1.3.3 Cytokine axes

As mentioned above cytokines such as TNFα, IFN-γ, IL-23, IL-17A, IL-17F, and IL-

22 seem to play a crucial role in psoriasis. There are two distinct axes that are recognized in the context of psoriasis, the older IFN-γ/TNF-α and the newer IL-23/T_H17 axes.

1.3.3.1 IFN-γ/TNF-α cytokine axis

IFN-γ was long considered to be the main cytokine in psoriasis pathogenesis and despite the emergence of the IL-23/IL-17 axis, it still remains a strong favourite[20]. It is the principal T_H1 cytokine and belongs to the family of type II IFNs[222]. The main producers of this cytokine are activated T_H1 cells, NK and NK T cells, as well as $CD8^+$ T cells. Supernatants from CD4+ T cell clones from psoriatic skin produce high levels of IFN-γ and GM-CSF[223]. Microarray studies have further underlined the importance of IFN-γ in psoriasis, showing its upregulation in psoriatic lesions, compared to healthy skin, as well as differential regulation of IFN-related genes[224]. In the same study transcription factor signaling alterations were the most consistent for STAT-1 and p48 (IFN-stimulated factor 3γ, both of which are induced by IFN-γ signaling pathway[20]. Interestingly, IFN-γ is known to have an antiproliferative effect on keratinocytes, but it was later explained by the fact that psoriatic keratinocytes have reduced activation of IRF1 and STAT-1 in response to IFN-γ[225]. At the same time IFN-γ upregulates adhesion molecules, chemokines, promotes further recruitment of lymphocytes, and stimulates DCs to produce IL-1 and IL-23[20].

TNFα has been historically grouped with IFN-γ, even though it is produced by many cell types including mast cells, macrophages, T_H17 and T_H22 cells[226-228]. There are two types of TNF, membrane bound and soluble versions. Both of these are biologically active and can bind either TNFR1 or TNFR2. The former is expressed on nearly all cells, while the latter is primarily found on endothelial and hematopoietic cells[229-231]. TNFR signaling can lead to NF-κB activation, resulting in proinflammatory signaling. Alternatively, the mitogen-activated protein kinase (MAPK) pathway is activated, leading to cellular differentiation, proliferation, or apoptosis[232,233]. Due to its complex proinflammatory effects on cells it is understandable why targeting TNF in autoimmune diseases such as psoriasis plays a beneficial role, but at the same time this could lead to a variety of side-effects[16,166].

1.3.3.2 The IL-23/IL-17 axis

In recent years IL-23 has been implicated as the major pathogenic cytokine in a variety of autoimmune diseases[89,95,234-238]. It is upregulated in psoriatic lesions[183] and has been shown to be downregulated following systemic anti-psoriatic therapy[166,239]. Moreover, increased number of T_H cells expressing higher levels of IL-23R have been found in the psoriatic lesions and general circulation of psoriasis patients[238]. The role of IL-23 in the pathogenesis of psoriasis has been underlined many times in different mouse models as well as in clinical trials, both of which will be described later on.

IL-23 is critical for differentiation[240,241] and production of IL-17 and IL-22 by T_H17[242] and other cells types[243]. However, at steady state IL-23R is rarely expressed by naïve T_H cells[244], but at the same time is thought to favour terminal differentiation of T_H17 cells, their maintenance and pathogenicity[245]. In humans T_H17 cells are quite heterogeneous and can also produce non-T_H17 cytokines such as IFN-γ[246,247].

There are numerous reports of IL-17-producing cells in psoriatic lesions[20,84,248,249], with increased levels of T_H17 cytokines in psoriatic, compared to non-lesional skin[250]. The role of T_H17 cells in psoriasis is further reinforced by the report of finding higher levels of these cells in the blood of psoriasis patients[86]. At the same time this was not the case for the serum of the patients[251-253]. There are also some studies that found $CD8^+$ and NK T cells producing IL-17 in the psoriatic skin[254,255].

The IL-17 family of cytokines is comprised of six members IL-17A-F[256,257]. At the same time their receptor family contains 5 receptor subunits IL-17RA-E[20,258]. Only IL-17A, C and F have been reported to play variable roles in psoriasis[259-261]. IL-17A and F signal through IL-17RA and the RC heterodimeric receptor, while IL-17C uses the IL-17RE subunit instead of RC[262,263]. These receptors are mainly expressed on macrophages, neutrophils and epithelial cells, such as keratinocytes[264-266]. IL-17A and IL-17F signaling can mobilize, recruit and activate neutrophils, which are very abundant within psoriatic plaques[267]. Moreover, IL-17A/F signaling in keratinocytes results in production of IL-6, antimicrobial peptides and chemokines, including CCR6 ligand CCL20[265,266,268,269].

IL-22 is another signature T_H17 cytokine, which is important for epithelial tissue homeostasis[270]. It is a member of the IL-10 cytokine family. IL-22 shares IL-10R subunit and has its own unique subunit IL-22RA1 for signaling. The unique subunit is expressed only by non-immune cells[271]. IL-22 signaling can also result in proinflammatory signaling and depends on target tissue as well as other cytokines[272-

[274]. Its role has been implicated in wound healing and tissue repair, but also in inflammation and infection[184,270]. In addition, IL-22 has synergistic effect with IL-17 in inducing production of antimicrobial peptides by keratinocytes, which are upregulated in psoriasis[275]. Increased levels of IL-22 mRNA have been found in psoriatic skin and in the sera of the patients, while after successful treatment of psoriasis its levels normalized[270]. Accordingly, acanthosis observed in psoriatic plaques is mainly attributed to IL-22 proliferative effect on keratinocytes[276].

Originally, T_H17 cells were thought to be the main producers of IL-22, but multiple studies were able to show that they account for less than 50% of IL-22 levels in psoriatic skin[277,278]. Moreover, IL-22 seems to have its own specialized transcription factor called aryl hydrocarbon receptor (AhR) [279], while T_H17 cells are mainly driven by receptor-related orphan receptor-γt (Rorγt)[209]. At the same time it is still a matter of open debate whether T_H22 cells represent distinct subset of T helper cells[226]. There have also been reports of $CD8^+$ cells producing IL-22 in psoriatic lesions[280]. The complex pathogenic cascade implicated in the pathogenesis of psoriasis that have been described so far is summarized in Figure 5[281].

1.3.4 Molecular mimicry theory

Despite psoriasis being considered an autoimmune disease no self-antigen has been identified to date. As described above the proposed mechanism of the disease initiation involves a complex interplay between a genetically susceptible individual and a variety of associated triggers, including infections, trauma, obesity, smoking, alcohol, medications, and anti-cytokine treatments for other non-related conditions[20]. But as with the majority of correlative findings it is hard to prove or disprove their real contributions. Recently, the LL-37-self-DNA, -RNA complexes were found to be a probable trigger for psoriasis[117]. However, this does not identify an antigen, which is recognised by the pathogenic T cells that are found in psoriatic lesions. One of the most prominent, well studied and accepted theories is the molecular mimicry theory, which implicates the homology between the streptococcal M protein and keratins 14, 16 and 17 to be the driving factor in the psoriasis pathogenesis[282].

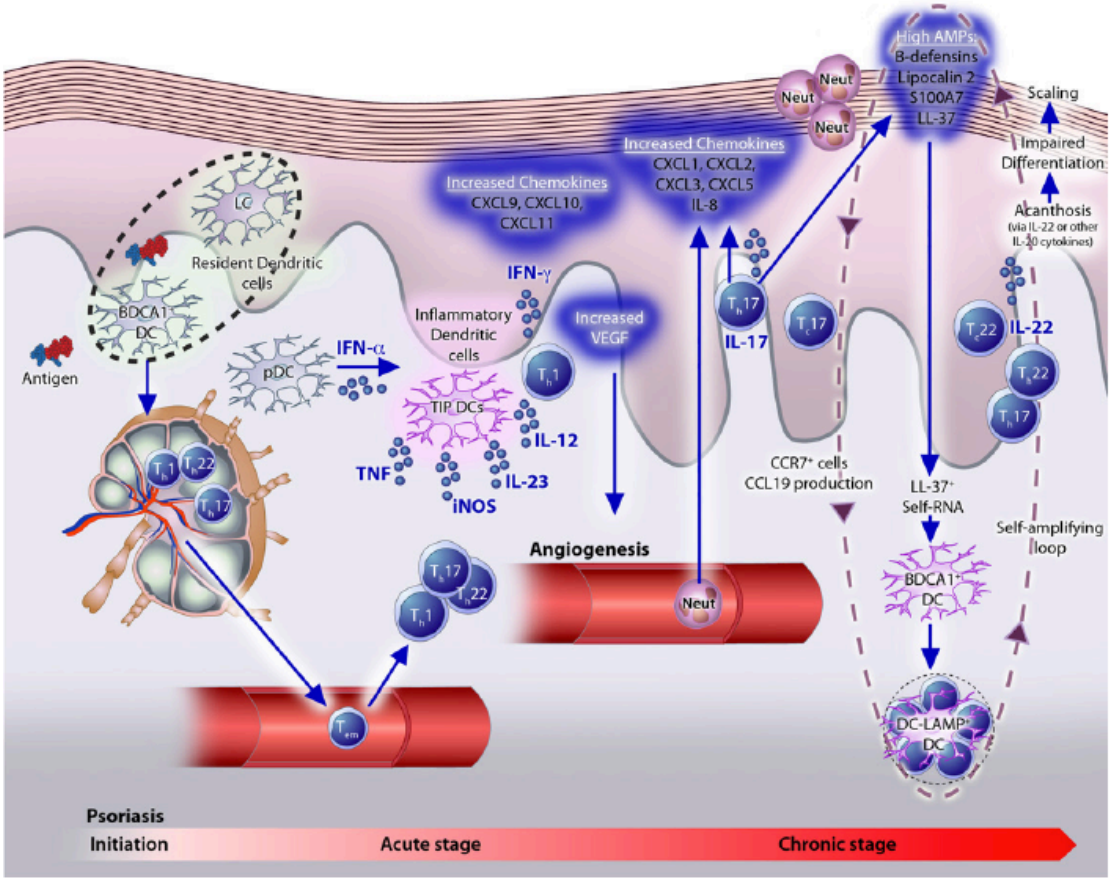

Figure 5. Initiation, acute, and chronic stages of psoriasis. pDCs produce IFN-α after being triggered by LL-37/self-DNA complexes, inducing maturation and differentiation of skin resident DCs. Inflammatory DCs and mDCs produce TNFα, iNOS, and IL-23, which induce T_H1 and T_H17 cell responses. The T_H1 cytokine IFN-γ induces keratinocytes to produce proinflammatory chemokines and increase production of vascular endothelial growth factor, promoting angiogenesis. IL-23 stimulates T_H17 and T_H22 cell differentiation and production of IL-17 and IL-22. IL-17 induces keratinocytes to produce chemoattractants for T cells, neutrophils, and mononuclear cells. IL-22 promotes epidermal acanthosis. IL-17 and IL-22 induce keratinocyte production of antimicrobial peptides, resulting in a self-amplifying inflammatory loop by inducing more LL-37/self-DNA complexes. Adapted from Guttman-Yassky et.al.,2011

1.3.4.1 Streptococcal infections and palatine tonsils

Correlation between streptococcal throat infection and the acute guttate form of psoriasis (an early onset form) has been documented in many studies. The incidence of streptococcal infections preceding this type of psoriasis ranges from 56% to 97%[283]. Moreover, chronic plaque psoriasis is generally exacerbated after streptococcal infections. Interestingly, these observations are consistent only with the three groups of β-hemolytic streptococci (A, C and G), all three of which express M-protein on their surface[110]. M-protein is a major virulence factor comprised of two polypeptide chains. It has a very variable N-terminal part, but highly conserved

membrane-anchored C-terminal, even within different bacterial strains and serotypes[42]. No association has been found between psoriasis and particular M serotypes. At the same time non-symptomatic carriage of streptococcus in the tonsil crypts is common.

1.3.3.2 Oligoclonality of lesional T cells in psoriasis

Numerous studies have reported chronic psoriasis lesions infiltrated by oligoclonal T cells[282]. Some follow-up studies show that dominant T cells clones persist in circulation for a long time, but reappear in psoriatic skin after drug-induced remission[284-287]. Moreover, identical clones have been identified in the psoriatic skin and synovial lesions of patients with psoriatic arthritis[288,289].

Recently, T cell clones, which expand in the palatine tonsils, were implicated in maintaining psoriatic lesions[290]. Accordingly, a number of patients with a history of moderate to severe plaque psoriasis all experienced at least 3 years remission after tonsillectomy. Interestingly, in all of them highly restricted T cell receptor (TCR) Vβ spectratypes of lesional T cells were found. Additionally, sequencing of the CDR3 of the TCR Vβ-chain found a number of identical clonal rearrangements within the lesional skin of all the patients[290]. Some of the identified clones were also found in the tonsils of these patients and were cutaneous lymphocyte antigen (CLA) positive, indicating the tonsils as a potential site for the expansion of the pathogenic T cell clones[282].

1.3.3.3 Cross-reactive T cells in psoriasis patients

Of more than 4000 mammalian proteins, human type 1 keratins have the highest homology with the streptococcal M6 protein[291]. This led to the theory that psoriasis is initiated by streptococcal superantigens and the disease is maintained by T cell clones that are cross-reactive for streptococcal M protein in the tonsils and keratins in the skin[292]. Further studies of the occurrence of circulating T cells that would be triggered by short homologous M and K peptides found much higher frequency of these cells in the psoriasis patients compared to controls[293,294]. Moreover, strong predisposition for psoriasis in carriers of the *HLA-Cw*0602* allele was utilized to study sets of homologous peptides from K17 and M proteins, which were also predicted to bind to HLA-Cw6. The HLA-Cw6 negative psoriatic patients had T cell responses to both

sets of peptides that were intermediate between those of the HLA-Cw6 positive patients and the controls. At the same time the majority of the responding cells were also positive for CLA[295]. Collectively these findings and the recent extensive study on remissions in psoriasis patients after tonsillectomy[296] make the cross-reactivity theory quite attractive and is most applicable to the group of early onset psoriasis patients[20,297].

1.4 Anti-psoriatic therapies

Even though the pathogenesis of psoriasis is not completely understood, some very effective and by now quite sophisticated therapies are available. These range from topical treatments for limited disease, to anti-biologics, which are used in cases of mild to severe psoriasis. Things that should be taken into account when prescribing the most efficient therapy are anatomical location, treatment history, exacerbating and favourable factors, quality of life concerns, etc[298].

1.4.1 Topical therapies

This type of therapy remains the most common type of psoriasis treatment and is generally used as monotherapy. It is primarily prescribed to patients with limited disease. Despite being effective for individual plaques, it is time consuming, and compliance with the treatment schedule is the greatest issue.

Topical corticosteroids are the most widely prescribed treatment for psoriasis worldwide. Depending on the potency of the agent, they can provide rapid efficacy, cosmetic acceptability, and are quite versatile. The Stoughton-Cornell classification is used to grade potency, and thus the relative effectiveness of corticosteroids on the basis of their ability to induce vasoconstriction[299]. It is proven that potent and very potent topical steroids are more efficacious for psoriasis treatment than the ones with mild or moderate potency[300]. The safety of long-term corticosteroid use is not yet defined. In one study, a 1-day per week corticosteroid application led to clinical response in 60% of patients, compared with 20% placebo controls, and was safe for up to 6 months[301].

Vitamin D_3 derivatives have recently become the first-line therapy for plaque psoriasis. They can be used either as a monotherapy or in combination with other treatments. The clinical responses to these are slower than with higher potency

corticosteroids, but their longer-term safety profile makes them valuable for maintenance therapy[302]. These analogues are very valuable in combination therapy, as they allow for a reduction in the dose and duration of other antipsoriatic agents[303].

Tazarotene is currently the only topical retinoid (vitamin A derivative) for the treatment of psoriasis. It has only moderate efficacy as monotherapy[304] and thus is mainly used in combination therapies[305]. In addition, it often causes skin irritation and cannot be used during pregnancy and it is advisable to limit its use in women of childbearing age.

Topical calcineurin inhibitors unlike corticosteroids do not cause skin atrophy, but their efficacy is quite low[306], unless specifically used on the thinner skin of the face, intertriginous areas, or genitals[307].

Dithranol was the main treatment for psoriasis for over 80 years, but its use has been declining steadily due to emergence of more cosmetically acceptable drugs. Additionally, it has lower efficacy than either topical corticosteroids or vitamin D_3 derivatives[300].

Coal tar, which is a mixture of many compounds, has been used in the treatment of psoriasis for over a century. Crude coal tar is the most effective form available and is slightly less efficacious than corticosteroids. Skin irritation, folliculitis, odour, and staining of clothing, and oncogenic potential limit its use[308].

1.4.2 PUVA photochemotherapy

PUVA photochemotherapy is the combination of an ingested psoralen photosensitiser and exposure to UVA. It is a very efficacious treatment for psoriasis, with a long lasting remission. But there are many undesirable side effects to this treatment, including nausea and headache from the ingested psoralen as well as skin burning, and photosensitivity. Premature cutaneous ageing, an increased risk of non-melanoma skin cancers, and possibly melanomas, are of particularly high concern[309,310]. After the introduction of narrowband UVB, the use of PUVA has substantially decreased worldwide.

1.4.3 Narrowband UVB

Natural sunlight is an effective treatment for psoriasis. The most effective wavelength is 311–313 nm (narrowband UVB) range[311]. Currently, narrowband (NB) or wideband

UVB is primarily used together with combinations of tazarotene, vitamin D3 analogues, or systemic treatments[312]. NB-UVB suppressed multiple parameters of the IL-23/IL-17 pathway in normalized psoriatic plaques, but not in nonresponsive plaques. Very recently, it was shown that narrowband UVB treatment decreases the numbers of TIP DCs, and their products, including inducible nitric oxide synthase (iNOS), IL-12/23p40, and IL-23p19. Moreover, during successful treatments NB-UVB suppressed IL-17 and IL-22 mRNAs, which strongly correlated with lesion resolution. Therefore, in addition to its known role in suppressing IFN-γ production, NB-UVB radiation therapy can also target the IL-17 pathway to resolve psoriatic inflammation[313].

1.4.4 Systemic non-biological treatments

Systemic treatments are primarily used for patients with moderate to severe disease and the ones that are unresponsive to topical agents or phototherapy. Issues that should be taken into consideration before prescribing such treatments include HIV status, presence of hepatitis, and previous systemic cancers. Most recently biological therapies provide new options for patients who previously were intolerant of or unresponsive to traditional systemic agents[298].

1.4.4.1 Retinoids

Oral retinoids are synthetic hormones that bind to nuclear retinoid receptors. By doing this they alter gene transcription in keratinocytes and possibly T cells, resulting in remission of psoriasis. Systemic retinoids are especially effective for the treatment of erythrodermic and pustular variants of psoriasis[314]. But as with many of anti-psoriatic drugs systemic retinoids should not be given to women considering pregnancy. It has recently been demonstrated that *all-trans* retinoic acid (ATRA) can suppress T_H17 cell differentiation and promote the generation of $Foxp3^+$ regulatory T cells via retinoic acid receptor signals[315].

1.4.4.2 Ciclosporin

Ciclosporin can be used as short-term treatment for moderate-to-severe psoriasis. It inhibits the calcineurin phosphatase-initiated activation of T cells[316] and may also exert a direct effect on keratinocytes[317]. Ciclosporin is extremely effective in inducing

rapid remission of psoriasis. Unlike methotrexate, ciclosporin is not teratogenic or myelosuppressive[318], but still requires very careful monitoring due to potential nephrotoxicity and hypertension[319,320].

1.4.4.3 Methotrexate

Methotrexate is a folic acid antagonist. It interferes with purine syntheses, which inhibits DNA synthesis and thus cell replication. Moreover, it has specific T cell suppressive activity. Even though newer and more sophisticated therapies are available by now, methotrexate continues to be affordable, gold standard treatment for recalcitrant psoriasis and psoriatic arthritis. Unfortunately, methotrexate has some severe side effects. Its use is completely out of question during pregnancy. Bone marrow suppression is the most common cause of death attributable to this treatment. During direct comparison between ciclosporin and methotrexate in treatment of psoriasis no significant differences in efficacy, time to remission, rates of remission, and quality of life improvements were observed[321].

1.4.4.4 Fumaric acid esters

Fumarates are naturally occurring compounds that can link the urea and citric acid cycles. Their benefits in psoriasis treatment are now considered to be mainly due to NF-κB inhibition and T-cell apoptosis[322]. **P**soriasis **A**rea and **S**everity **I**ndex (PASI) reductions associated with fumarates are similar to those of methotrexate and ciclosporin[323,324]. Despite absence of severe adverse events, the fumarates can cause highly unpleasant gastrointestinal symptoms and fatigue[325].

1.4.5 Biologicals

Better understanding of the immune pathways involved in the pathogenesis of psoriasis has led to the development of new agents that specifically target them. The coincidental alleviation of psoriasis that occurred during the treatment of other inflammatory diseases identified the involvement of T cells in psoriasis. This resulted in the development of anti–T cell therapies. Most recently, greater appreciation of the molecules involved in the pathogenesis of psoriasis resulted in cytokines and their receptors being targeted. As with all therapies these days, advancing technological platforms and the availability of high-throughput sequencing will hopefully allow

personalized immunogenetic therapies. The eventual goal of this would be to predict individual prognosis and therapeutic response with minimal side effects.

1.4.5.1 Anti-T cell biological therapies

Alefacept was the first biological agent approved for the treatment of psoriasis. It is a human recombinant protein that binds to CD2 on memory effector T cells, selectively interfering with the function of APCs and thus, T-cell activation[326]. It also causes apoptosis of memory-effector $CD45RO^+$ T cells in the skin[327]. Treatment with Alefacept resulted in 20% of patients achieving a 75% reduction in PASI (PASI 75) after 12 weekly intramuscular injections and had hardly any associated side-effects[328]. Efalizumab is a humanized monoclonal antibody, which binds CD11a and inhibits activation of T cells, as well as their adhesion to endothelial cells, thus preventing circulating T cells from entering the skin[329]. Efalizumab is slightly more efficacious than Alefacept and substantially improved psoriasis in patients with moderate-to-severe disease. About 25% of patients achieved PASI 75 by 12 weeks for periods of up to 15 months. The maximum response (47% PASI 75) was observed after about 24 weeks of treatment without substantial side effects[330-332]. Unfortunately, Efalizumab was recently withdrawn from the European and American markets after five years of use due to three cases of progressive multifocal leukoencephalopathy[333].

1.4.5.2 Anti-TNF therapies

Etanercept is a human recombinant TNF receptor p75 protein that binds to TNFα and β. It can be self-administered subcutaneously, with 34% of patients achieving PASI 75 by 12 weeks. Higher doses of 50 mg twice weekly, as approved in the USA, for up to 12 weeks of initial therapy, resulted in 49% of patients achieving PASI 75[334]. Very importantly, etanercept also relieves fatigue and symptoms of depression in patients with moderate-to-severe psoriasis. Moreover, Etanercept is also highly efficacious for psoriatic arthritis treatment, with a reduction in the signs and symptoms of joint disease in 73–87% of patients after 12 weeks of treatment[335]. Interestingly, effective treatment of psoriasis with etanercept was recently linked to suppression of IL-17 signaling rather than immediate response TNF genes[336,337].

Infliximab is a chimeric monoclonal antibody, which neutralises the activity of TNFα. It is more efficacious than etanercept with 82% of patients achieving PASI 75 after 10

weeks of treatment[338]. Infliximab is also quite effective for treatment of psoriatic arthritis[339].

Adalimumab is a fully human, anti-TNFα monoclonal antibody. Its efficacy is in-between etanercept and infliximab with 54% of patients achieving PASI 75 after 24 weeks[340]. As with the other two anti-TNF therapies it also has positive impact on psoriatic arthritis[341].

1.4.5.3 Anti-IL-12/23p40 therapies

Ustekinumab is a human IgG1 monoclonal antibody, which binds to the p40 subunit of IL-12 and IL-23 and prevent its interaction with IL-12Rβ1[342] (see Figure 3). It was shown to be more efficacious than etanercept, with improvement to PASI 75 at week 12 in 67.5% of patients who received a low dose of ustekinumab and 73.8% of patients who received a higher dose, compared with 56.8% of those who received etanercept[343].

Briakinumab is a similar monoclonal antibody, which seems slightly more efficacious than ustekinumab with 80.7% of patients achieving PASI 75 at week 12[344]. However, currently only ustekinumab is approved for treatment of psoriasis.

1.4.5.4 Anti-IL-17 therapies

Currently no anti-IL-17 therapies are approved for treatment of psoriasis. However, recent clinical trials of three drugs targeting IL-17 have shown great promise. Secukinumab is a human monoclonal antibody against IL-17A[345]. Ixekizumab is a humanized monoclonal antibody against IL-17A[346]. Brodalumab is a human anti–IL-17RA monoclonal antibody[347]. Interestingly, these antibodies have shown quite variable results in clinical trials. Ixekizumab has proven to be the most efficacious with 82% of patients achieving PASI 75[346]. At the same time this was significantly lower for Secukinumab (40%)[345] and Brodalumab (45%)[347]. These results have at least two possible explanations. In case of Ixekizumab it seems that the efficacy of ustekinumab is purely due to blocking IL-23 and thus shows that the IL-23/T_H17 axis is a lot more important in psoriasis than IFN-γ axis. At the same time, the results for Secukinumab would imply a synergistic role of IL-17 and IFN-γ axes. Finally, the results for Brodalumab suggest the same as Secukinmab, but it is known that IL-17A, C, E and F signal through IL-17RA[256], which complicates the interpretation of these

results. However, Ixekinzumab results show great promise and prove that due to our better understanding of the pathogenesis of psoriasis more efficacious and specific therapies are being developed.

1.5 Models of psoriasis

With the exception of a few rare cases in primates, psoriasis is unique to humans. Therefore a big part of the described mechanism of psoriasis pathogenesis (Figure 5) would not have been possible without preclinical model systems The available models of psoriasis generally provide only an approximation of the disease[16,202-205,348,349]. The four main types of *in vivo* animal models nearly exclusively rely on mice, with variable experimental settings: spontaneous mutation, genetic engineering, xenotransplantation and cytokine injection models. However, major differences are quite apparent between human and mouse skin, making the results of the model studies less conclusive. Firstly, the human skin is thicker in comparison with that of mice. Secondly, mice have much higher average hair distribution density. Mice do not have sweat glands and melanocytes in the interfollicular epidermis, but have more rapid epidermal turnover and the presence of a unique epidermal subset of γδ T cells[350] (Figure 6)[205].

1.5.1 Spontaneous models

There are some mouse strains that develop spontaneous skin diseases, which are macroscopically reminiscent of psoriasis. Chronic proliferative dermatitis disease is observed in mice with *Sharpin*^{cpdm}/*Sharpin*^{cpdm} allele mutation. These studies have shown increased rate of keratinocyte proliferation, but primarily granulocytes and only a small fraction of T cells comprised the skin infiltrating leukocytes. However, in this model, immune cells infiltrates were also observed in some joints. At the same time the disease could not be transferred by bone marrow or spleen transplants. Treatment of these mice with corticosteroids, resulted in nearly complete regression of the lesions, but this was not the case for systemic cyclosporine treatment[351].

The flaky skin mutation (*Ttc7*^{fsn}/*Ttc7*^{fsn}) mice displayed a marked dermal infiltration of lymphocytes and mast cells, together with a small increase in the number of granulocytes. Some angiogenesis, as well as progressive papillomatosis were also observed in this model[352]. Another spontaneous psoriasis-like mouse strain is

homozygous for the mutated asebia allele (*Scd1*^{ab}/*Scd1*^{ab}) [353]. Overall, these models have many features reminiscent of psoriasis, but they lack T-cell infiltrates, characteristic of psoriatic lesions and their responses to treatment with antipsoriatic drugs are low[205,354].

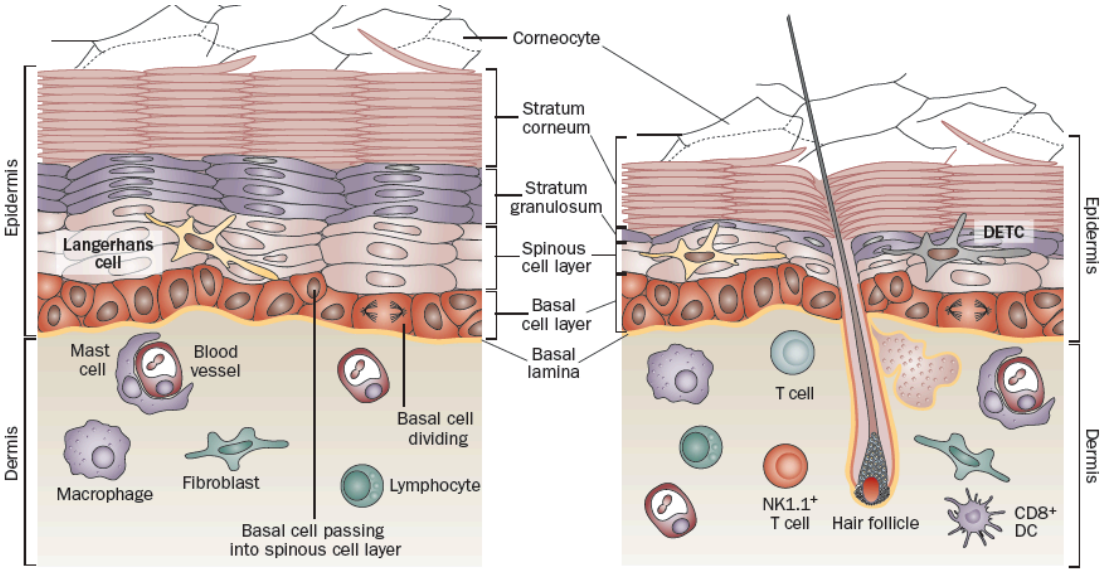

Figure 6. Schematic representation of human and mouse skin showing differences in stratification and the different cell types present.
Adapted from Wagner et.al., 2010.

1.5.2 Genetically engineered mouse models

There are two broad types of genetically engineered mouse models of psoriasis: mice that had a genetic element introduced (transgenic mice), and the ones in which a genetic element had been removed (gene knock-out mice). These models are very good for determining if overexpression of a given cytokine, growth factor, or adhesion molecule in isolation would have a role in skin inflammation[16]. Epidermal overproduction of molecules expressed under the control of promoters that act in the basal epidermal layer: keratin 5 (K5) and keratin 14 (K14), as well as proteins that function in the suprabasal epidermal layers, including involucrin and keratin 10 (K10), induce the development of a psoriasis-like disease in several mouse models[205].

1.5.2.1 Gene knock-out models

As described above IL-1β is constitutively expressed in keratinocytes and its mRNA is increased in psoriatic lesions[355]. In line with this, mice with a targeted deletion of the IL-1 receptor antagonist gene also develop a psoriasis-like phenotype with epidermal hyperproliferation and an inflammatory infiltrate in the skin, as well as some joint inflammation[356].

The NF-κB pathway is important for transcription of IL-1 and many other proinflammatory genes (see above). Deletion of one of the subunits of the NF-κB inhibitor, IκB kinase IKK2, induced a strong psoriasis-like inflammation in the mouse skin. This inflammation was dependent on TNF with marked infiltration and activation of dermal macrophages. But at the same time these mice had developed features uncharacteristic for psoriasis, such as keratinocyte apoptosis and was T-cell-independent[357,358].

The transcription factor AP-1 members have been shown to be expressed in human and mouse epidermis[359]. Skin specific deletion mouse models show quite conclusively that some of these are important regulators of skin inflammation[360,361]. Inducible epidermal deletion of JunB and c-Jun in adult mice leads to a chronic psoriasis-like disease, including joint involvement[362]. Moreover, these mice had increased production of IL-23 and T_H17 cytokines. However, in the same model the severity of inflammation was reduced in mice crossed with the ones lacking either *RAG1* or the *TNFR1* genes, suggesting that cells other than T cells initiate the inflammation. At the same time, a therapeutic approach using an anti-VEGF antibody led to an improvement in skin inflammation[363].

1.5.2.2 Transgenic mouse models

STAT3 is crucial for the differentiation of T_H17 cells. At the same time, it has importance for wound healing and skin carcinogenesis[364,365]. In the K5-STAT3C mouse model, skin inflammation reminiscent of psoriasis develops in a spontaneous fashion or can be triggered. It has features very similar to psoriasis, including loss of the stratum granulosum, dilated blood vessels and an immune infiltrate of lymphocytes and neutrophils[364].

As described above for the K5-JunB/c-Jun model, VEGF was important for the disease development and is also elevated in the skin and serum of patients[49].

Accordingly, K14-VEGF transgenic mice develop psoriasis-like skin phenotype, with increased proliferation and abnormal differentiation of keratinocytes, and an immune infiltrate similar to psoriatic lesions[366].

Anti-TNF therapy is efficacious in psoriasis[20,334]. Accordingly K14-TNF mice have an immune infiltrate in the skin, but these also die of intestinal and liver necrosis[367]. Interestingly, peroxisome proliferator activator receptor β/δ (PPARβ/δ), which regulates keratinocyte differentiation, is a downstream target of TNF and has been implicated in psoriasis[368]. Suprabasal induction of PPARβ/δ caused a skin inflammation in mice, which was dependent on STAT3 phosphorylation, IL-12/23p40, and TNF. It also simulates the features of psoriasis in relation to signaling pathways[369].

Finally, in line with IL-12/23p40 and TGF-β being important for differentiation of T_H17 cells[139,242], K5-TGF-β1 and K14-p40 models also closely resemble psoriasis[370,371].

1.5.3 Transplantation models

Transplantation models are the most sophisticated models of psoriasis as they involve transferring the disease from mice or humans into mice. Therefore they duplicate the transfer of the disease as it has been described for the human disease[200,201]. Moreover, these models could be the best types of models to conduct preclinical tests of potential anti-psoriatic drugs.

1.5.3.1 Cell transplantation models

A disease with psoriasis features could be transferred into $Prkdc^{scid}$ mice with $CD4+CD45RB^{hi}$ T cells from donors that were MHC matched, but mismatched for minor histocompatibility antigens[372]. Additionally, *RAG2*$^{-/-}$ mice reconstituted with $CD4+CD45RB^{hi}$ T cells developed quite similar phenotype[373]. Both of these models support the notion that psoriasis-like skin lesions can be induced by T cells, but at the same time run into danger of mimicking graft vs. host disease instead.

1.5.3.2 Xenotransplantation models

In this model the skin from psoriasis patients is transplanted onto immunosuppressed

mice. Interestingly, transplants can be from non-lesional or lesional skin. These can be employed to study either the development of psoriatic lesions or already established psoriasis. Therefore, they are well designed to understand the disease initiating pathways, as well as disease maintaining ones. The most recent model of this type uses AGR129 (*RAG*$^{-/-}$, *IFNRI*$^{-/-}$, *IFNRII*$^{-/-}$) mice as hosts. This model has been invaluable for underlining the importance of T cells, TNF, pDCs, type I IFNs, α1β1 integrin and IL-23 in the pathogenesis of psoriasis[76,238,374,375].

1.5.4 Other models

Despite marked success of the xenotransplantation model in advancing our understanding of psoriasis and development of anti-psoriatic therapies, not all of the findings in this model have been successfully translated into effective anti-psoriatic treatments. Most recently, this was underlined by failure of anti-IFN-α therapy in psoriasis trials[376]. This and other factors, such as limited availability of human skin grafts from psoriasis patients, cumbersome experimental procedure and a long time for the disease development have led to the development of new models. These were primarily aided by our increasing understanding of the disease pathways and clinical observations.

This type of model utilizes our knowledge of the main molecular players in psoriasis pathogenesis. This is used to 'shortcut' the original stimulus for the disease initiation. Subcutaneous injections of Il-21, Il-23, and to a lesser extent Il-12, induce a psoriasis-like disease in mice[377-381].

1.5.5 Aldara model

The Aldara model is based on clinical observations that topical treatment with Aldara cream, for unrelated conditions, could trigger and cause relapses of psoriasis in patients[170-174]. The active ingredient of Aldara, Imiquimod is a ligand for TLR7, and a strong immune modifier. In clinics, it is used for topical treatment of genital and perianal warts, caused by human papilloma virus (HPV)[382]. Recently, the clinical applications were additionally expanded to other virus-associated skin abnormalities and cancerous skin lesions, such as actinic keratoses[383] and superficial basal cell carcinomas[384]. Additionally, Aldara induced psoriatic lesions were found to contain pDCs, similar to that of lesions caused by other triggers[170]. Moreover, this model

satisfies most criteria for a good psoriasis model[203], including: epidermal changes based on keratinocyte hyperproliferation and altered differentiation; papillomatosis; presence of inflammatory cells, including T cells, DC, and neutrophils; a functional role for T cells; and altered vascularisation[385]. The only criterion that was not been tested in this model was response to the well-established antipsoriatic drugs. It was also shown that the Aldara skin inflammation in mice is dependent on IL-23p19 and IL-17RA. What also makes this model very attractive is the quick development of the psoriasis-like lesions, within 4-5 days. On the other hand it implies innate immune mechanisms being pivotal for the development of Aldara driven skin inflammation. Finally, in the original study it was mentioned that higher percentage of IL-17A producing γδ T cells were detected in the in spleens of Aldara-treated mice compared with controls[385].

1.6 Innate sources of IL-17 and IL-22

Recent studies on the role of IL-23 in autoimmunity unearthed a novel subset of T cells that are important for chronic inflammation and tissue damage[139,208]. This led to the quickly accepted T_H17 cell paradigm, in which IL-6–STAT3 activation pathway of the transcriptional regulator retinoic acid RORγt controls the lineage fate of IL-17A-, IL-17F-, IL-21- and IL-22-producing T cells (known as T_H17 cells)[386]. These are highly responsive to IL-1 receptor 1 (IL-1R1) and IL-23R signaling[209,387]. But this cell lineage cannot explain the early IL-17-dependent responses, crucial during stress responses and host defense. It has been observed that the IL-17-mediated pathway is triggered within hours after epithelial cell injury or through PRRs[388,389]. This would clearly not provide enough time for the development of T_H17 cells[386].

Further support of the innate sources of IL-17 and IL-22 is underlined by studies where these cytokines were induced in *RAG*$^{-/-}$ mice[245,390,391]. A closer look at evolution also reinforces the idea that IL-17 is an innate cytokine as ancestors of this gene-family are preserved among invertebrate species[392]. Finally, studies in IL-23R, RORγt, IL-17A and IL-17F reporter and fate-mapping mice clearly established that at least IL-17 is primarily produced by CD4 and CD8 negative T cells[244,393-395].

1.6.1 γδ T cells

Together with B cells, αβ T cells, γδ T cells and NK T cells are the only cells that use somatic gene rearrangement to generate diverse antigen receptors. This cell type was simultaneously classified by two groups[396,397] and other nomenclatures have been suggested as well[398]. Here the Heilig and Tonegawa nomenclature is used[397]. γδ T cells constitute only up to 5% of the circulating lymphocytes in most adult animals[399]. However, they are a lot more abundant in the epithelial tissues, such as the skin, intestine and reproductive tract, where they can make up to 50% of all T cells. In the thymus, γδ T cells develop from double-negative thymocytes and branches off from αβ T cells at the transition of thymocytes from the DN3 to the DN4 stage. Models of selective and instructive αβ vs. γδ lineage commitment have been postulated based on TCR signal strength[400] or Notch signaling[401]. Current knowledge favours the signal strength hypothesis[402,403]. TCRγδ$^+$ thymocytes are first ones to develop out of all T cells and can be found in the embryonic thymus starting from embryonic day 14. The waves of γδ T cell development start with Vγ5$^+$Vδ1$^+$ DETCs, followed by Vγ6$^+$Vδ1$^+$ progenitors that subsequently localize to the mucosal tissues. Thymic terminal transferase is not expressed during these prenatal stages, resulting in simple V-D-J joins that characterise the canonical TCRs of fetal-derived γδ cells[398,403]. On the other hand, postnatal gut and lymphoid γδ T cells acquire diverse V-D-J joins in their Vγ1, 2, 4 and 7-containing TCRs[403]. This ordered progression of γδ T cell generation and tissue-specific localisation are partly due to ordered Vγ-region transcription and rearrangement and partly due to preTCR programming (Figure 7)[399,403,404].

Peripheral γδ T cells can recognize self and non-self ligands, which divides them into antigen experienced and antigen naive groups, which produce IFNγ or IL-17, respectively[405]. At the same time, this differentiation can also be made based on surface marker CD27 (TNFR family member), with CD27$^+$ cells producing IFNγ and CD27$^-$ ones producing IL-17[406]. Similar distinction is achieved by using markers such as CCR6, NK1.1 and SCART2[407,408]. Unlike naïve αβ T cells, certain subsets of γδ T cells are programmed for rapid responses to a variety of stimuli by expressing multiple PRRs (TLR1 and 2, dectin-1) through production of IL-17 and IL-22[409].

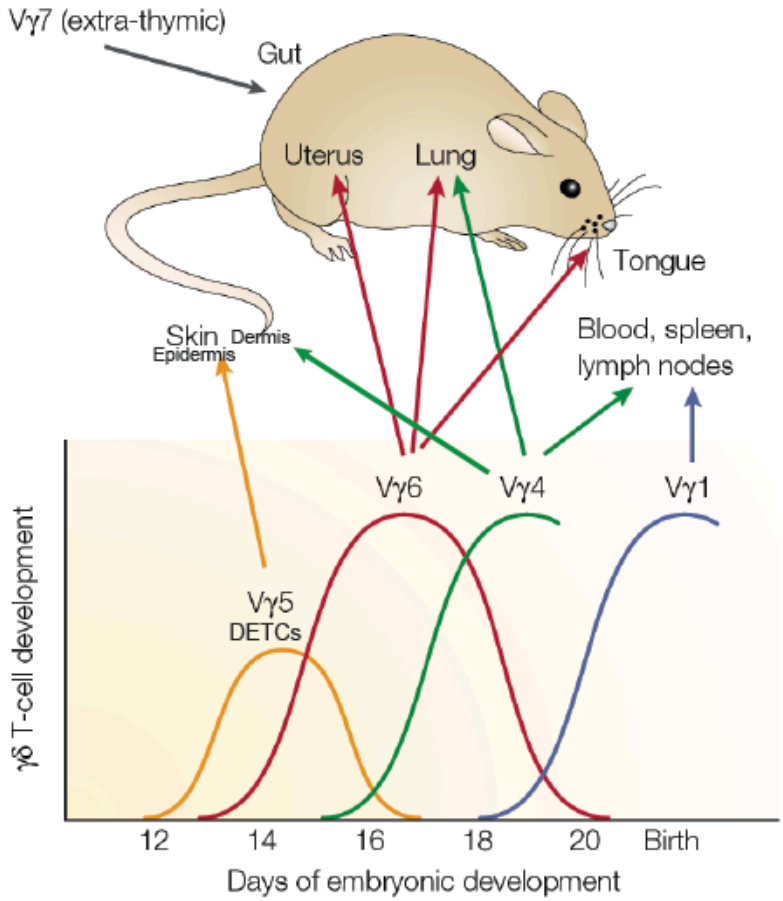

Figure 7. Mouse γδ T-cell generation is developmentally programmed. γδ T cells are encoded by specific *V*γ-gene segments (see graph) and migrate from the thymus at defined periods of fetal and neonatal development. Modified from Carding and Eding 2002.

At the same time IL-17-producing γδ T cells constitutively express IL-1R and IL-23R, triggering of which amplifies their production of cytokines[410]. Recently, γδ T cells were shown to be important for protection against a variety of pathogens, including *Listeria*, *Streptococcus* and *Malaria*[411-413]. Moreover, they have been implicated to play crucial roles in a variety of mouse models of autoimmunity[410,414-417]. Recently, IL-17-producing populations of γδ T cells have been found in the skin of mice[418,419]. Finally, there has been a report that the numbers of γδ T cells are increased in psoriatic lesions[420].

1.6.2 Innate lymphoid cells (ILCs)

Very recently a new innate source of IL-17 and IL-22 was identified in form of innate lymphoid cells (ILCs)[421]. These develop from the same hematopoietic precursors as T

lymphocytes, but do not express any specific antigen receptors[422]. One of the more well studied members of this family are the NK cells. NK cells are critical for the immune recognition of transformed or tumour cells as well as cells infected with viruses[67]. Another prominent member of this family are lymphoid tissue inducer (LTi) cells that are important for the development of the lymphatics[423].

All ILCs depend on transcription factor Id2 (inhibitor of DNA binding-2). ILCs do not express any known lineage markers, but are positive for IL-7 receptor α chain (IL-7Ra; CD127) and the cytokine common gamma (γc) chain[424]. Originally, these cells were identified as a very minor population within the spleen, which were readily able to produce IL-17 and IL-22[421]. Moreover, they were found to constitutively express RORγt, AhR, CCR6 and IL-23R, strikingly resembling IL-17-producting γδ T cells[88,421]. The majority of studies have shown that IL-22-producing ILCs are particularly important in the intestinal homeostasis as well as protection against certain bacterial strains[425], viruses[426], and tumour rejection[427]. Importantly, their role was recently demonstrated in the models of colitis[428-430]. However due to their low abundance and lack of specific markers these cells are hard to study and are still quite poorly characterised.

1.7 Aims of the study

The original study on Aldara psoriasis was primarily performed using Balb/c mice[385]. Therefore, the first objective would be to establish the Aldara mouse model of psoriasis in C57/B6 wild-type mice. Aldara cream is a kind of a 'black box'. Thus, it would be important to understand if the inflammation observed in this model is purely dependent on the TLR7 agonistic activity of active ingredient of Aldara, Imiquimod[431]. pDCs are the cell type that expresses the highest levels of TLR7. Therefore, their role and the role of their effector cytokines type I IFNs need to be established in the initiation of the Aldara psoriasis. This model has been quite well characterized and shown to satisfy the majority of the criteria for a good psoriasis model[203]. The only criterion that has not been addressed was the responsiveness to conventional ant-psoriatic therapies, and therefore will be tested in this study.

Moreover, in the original study only the dependence on IL-23p19 and IL-17RA were shown[385]. Thus, it would be interesting to establish the differential roles of so called T_H17 cytokines IL-17A, IL-17F and IL-22, as well as transcription factor RORγt in the Aldara psoriasis model. As discussed above the Aldara induced skin inflammation develops very rapidly and therefore it would be of particular interest to study the effector cells that are crucial for the development of the disease. It seems quite unlikely that T_H17 would be the only cell type to play an important role in the Aldara psoriasis and therefore the roles of γδ T cells and may be ILCs need to be investigated. Overall, better understanding of the disease initiating events in this model would help improve our understanding of the initiation phase of the pathogenesis of psoriasis.

2 Results

2.1 The Aldara psoriasis model characterisation

2.1.1 Appearance of Aldara psoriasis

To initiate psoriasis-like inflammation, Aldara cream was applied to the shaved back and the ears of C57BL/6 mice for 6 consecutive days as previously described for BALB/c mice[385]. From day 3 onwards we observed significant thickening (acanthosis), reddening (erythema) and scaling (dysregulation of keratinocyte differentiation) of the portions of skin treated with Aldara, compared to the ones treated with control cream (Figure 8A). Overall, the macroscopic appearance of the disease replicated human psoriasis quite well.

2.1.2 Kinetics of Aldara psoriasis

Originally it was intended to calculate a cumulative PASI-like score for every experiment. However, this was hard to replicate, mainly due to mice having variable amount of hair after shaving, which affected the redness parameter. It was also quite hard to use scaling as a reliable readout, due to mice scratching and biting each other. To circumvent this and to monitor the course of the disease daily, only the thickness of the ears and the back skin were measured. Additionally, the mass of the mice was also measured every day. To adjust the measurements so that the results could be replicated in other experiments percentage change in all parameters were calculated.

The clinical courses of the inflammation of the ear and the back skin were overall very similar but displayed slightly different kinetics. The back skin seemed to get inflamed earlier and the peak of inflammation was around Days 4 and 5. At the same time, the peak of ear inflammation was on Day 6 (Figure 8B). Another interesting feature, which was observed during treatment with Aldara, was that mice lost up to 15% of their body mass by Day 2, but at Day 6 they generally recovered to their starting mass.

2.1.3 Histological features of Aldara psoriasis

Histological analysis of the Aldara-treated back skin revealed inflammation from Day 2 onwards, which increased in severity on Day 3, and decreased from Day 4 on. The ears showed very similar inflammatory pattern of inflammation, but its apex of

inflammation was reached by Days 4 to 5. Moreover, Aldara treatment resulted in hyperproliferation of keratinocytes and disturbed epidermal differentiation as indicated by acanthosis and hyperparakeratosis. In the dermis, a massive infiltrate of leukocytes could be observed (Figure 8C).

To verify the presence of the neutrophils in the Aldara treated skin myeloperoxidase (MPO) staining was performed. This clearly highlighted the accumulation of neutrophils in the parakeratotic stratum corneum and was highly comparable to Munro microabscesses in human psoriasis. (Figure 8C).

2.1.5 Aldara psoriasis model responds to anti-IL-12/23p40 treatment

As discussed above, this model satisfies most criteria of a good psoriasis model[203,385]. The only criterion that had not been tested in this model was the response to the well-established antipsoriatic drugs. Ustekinumab is a monoclonal antibody against human IL-12/23p40, which had great success in clinical trials (73% PASI 75 at 12 weeks)[432], and by now is the only drug of this type approved for treatment of psoriasis. To see if the Aldara model would be responsive to anti-IL-12/23p40, the mice were given a single intra peritoneal injection of 200 µg of the antibody on Day 2. Surprisingly, this was very successful as the mice that received the anti-IL-12/23p40 antibody had significantly lower inflammation compared the mice that received an isotype control antibody (Figure 8E). From this point onwards only the kinetics for the back skin thickness will be described due to strong similarities between the disease courses in the back skin and the ears.

This finding and the recent report that Aldara psoriasis is responsive to NB-UVB therapy[433] indicate that Aldara psoriasis model satisfies all of the criteria for a good psoriasis model[203]. These include epidermal changes based on keratinocyte hyperproliferation and altered differentiation; papillomatosis; presence of inflammatory cells, including T cells, DC, and neutrophils; a functional role for T cells; altered vascularization; and responsiveness to anti-psoriatic treatments. Additionally, this finding is in line with the earlier report where Aldara skin inflammation was dependent on IL-23p19[385].

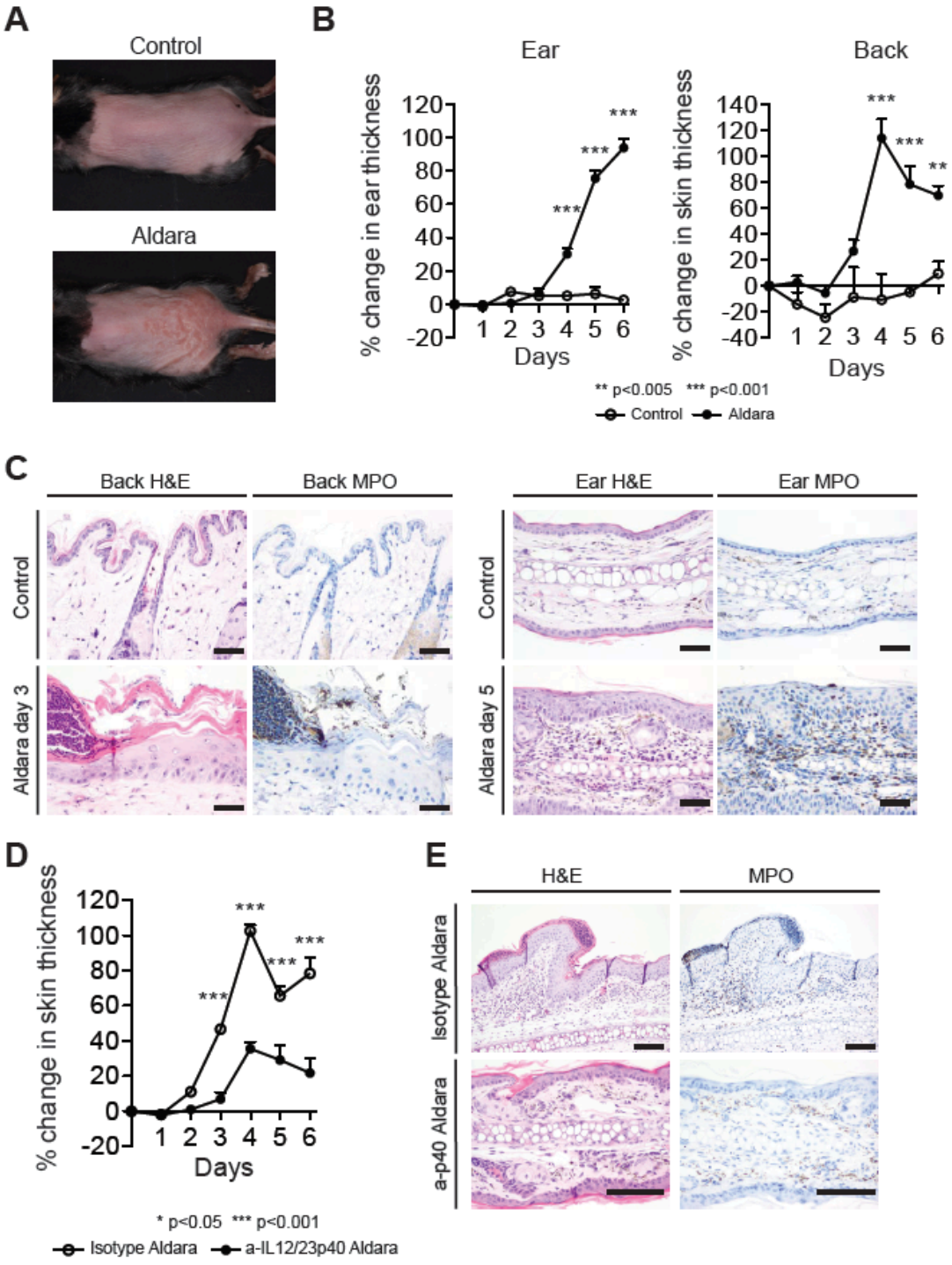

Figure 8. The Aldara psoriasis model characterisation. (**A**) Images show a representative back skin of a WT mouse treated for 5 days with Aldara or control cream. (**B**) Kinetics of Aldara-induced skin inflammation was quantified over 6 days as percentage increase in the thickness of ear (left) and back (right) skin. Data are shown as the mean percentage ± SEM (n=4). (**C**) Back (day 3) and ear (day 5) skin sections of Aldara treated vs. control treated mice were stained with H&E and for anti-MPO. Original magnification: x40 Scale bar: 50 µm. (**D** and **E**) WT mice were treated with Aldara and anti–IL-12/23p40 mAb or isotype control on day 2. (**D**) Kinetics of skin inflammation as percent increase in thickness over 6 days (n = 4). (**E**) Skin sections were stained with H&E and anti-MPO. Scale bars: 100 µm.

2.2 The signaling pathways involved in Aldara psoriasis

As mentioned above the active ingredient of Aldara is Imiquimod, which is a strong TLR7 and a weak TLR8 agonist. Currently, it is believed that mouse TLR8 is nonfunctional. However, Imiquimod itself has other activities such as interference with the adenosine signalling[434] and direct apoptotic activity[431].

2.2.1 Aldara psoriasis is not completely dependent on TLR7

To elucidate the mode of action of Aldara, *tlr7-/-* and *myd88-/-* mice were treated with the cream. Both mutant mouse strains did not develop psoriasiform lesions, as they completely lack leukocyte infiltration into the tissue and inflammation, unlike the wild-type counterparts. At the same time only the lack of MyD88 resulted in an almost complete absence of Aldara activity, as seen by the absence hyperproliferation of keratinocytes upon cream treatment. TLR7 deficiency does allow such a hyperproliferatory response to develop, but it does not lead to inflammation (Figures 9A and B). Thus overt TLR7 signalling seems to be the main immunologic trigger of psoriasiform plaque formation in the Aldara psoriasis model, but TLR-7/MyD88 independent Aldara activity was also confirmed. Furthermore, MyD88 is an adaptor molecule in a variety of pathways, including the signalling of IL-1 and IL-18 receptors[131,435,436], which could be the reason for the differential phenotypes in the two mouse strains[437]. Finally, this could also be the result of additional active ingredients in the Aldara cream formulation (M. van den Broek, unpublished observations).

Another interesting observation was that the mice lacking TLR7 or MyD88 were resistant to the weight loss due to Aldara treatment, unlike wild-type mice (Figure 9C). Therefore, it seems that the systemic effects of Aldara are mediated exclusively through the TLR7 agonistic activity of Imiquimod.

2.2.2 Type I IFNs do not play a role in Aldara psoriasis

As described above pDCs are a small DC subset circulating through peripheral blood and secondary lymphoid organs. They represent key innate effector cells during antiviral immune responses due to their capacity to secrete large amounts of type-I IFN upon TLR7/9 stimulation[160]. There is plenty of evidence that pDC-derived IFN-I signaling is an upstream event preceding autoimmune inflammation and psoriasis development[76,117,374]. However, these findings were not supported in psoriasis clinical trials targeting IFN-α.

To test if type I IFNs play a role in the Aldara psoriasis, *IFNAR*$^{-/-}$ mice were compared to the wild-type mice. Interestingly, there was no difference between the two mouse strains macroscopically as well as histologically (Figures 9A and B). This points at pDCs and their effector cytokines type I IFNs not playing a role in Aldara psoriasis.

On the other hand, *IFNAR*$^{-/-}$ mice were still resistant to the weight loss, previously observed in *tlr7*$^{-/-}$ and *myd88*$^{-/-}$ mice (Figure 9C). This implies that the TLR7 agonistic activity of Imiquimod in the Aldara cream is partially responsible for the observed inflammation, but primarily mediates the systemic effects of the Aldara cream through TLR7-MyD88-IFN I pathway.

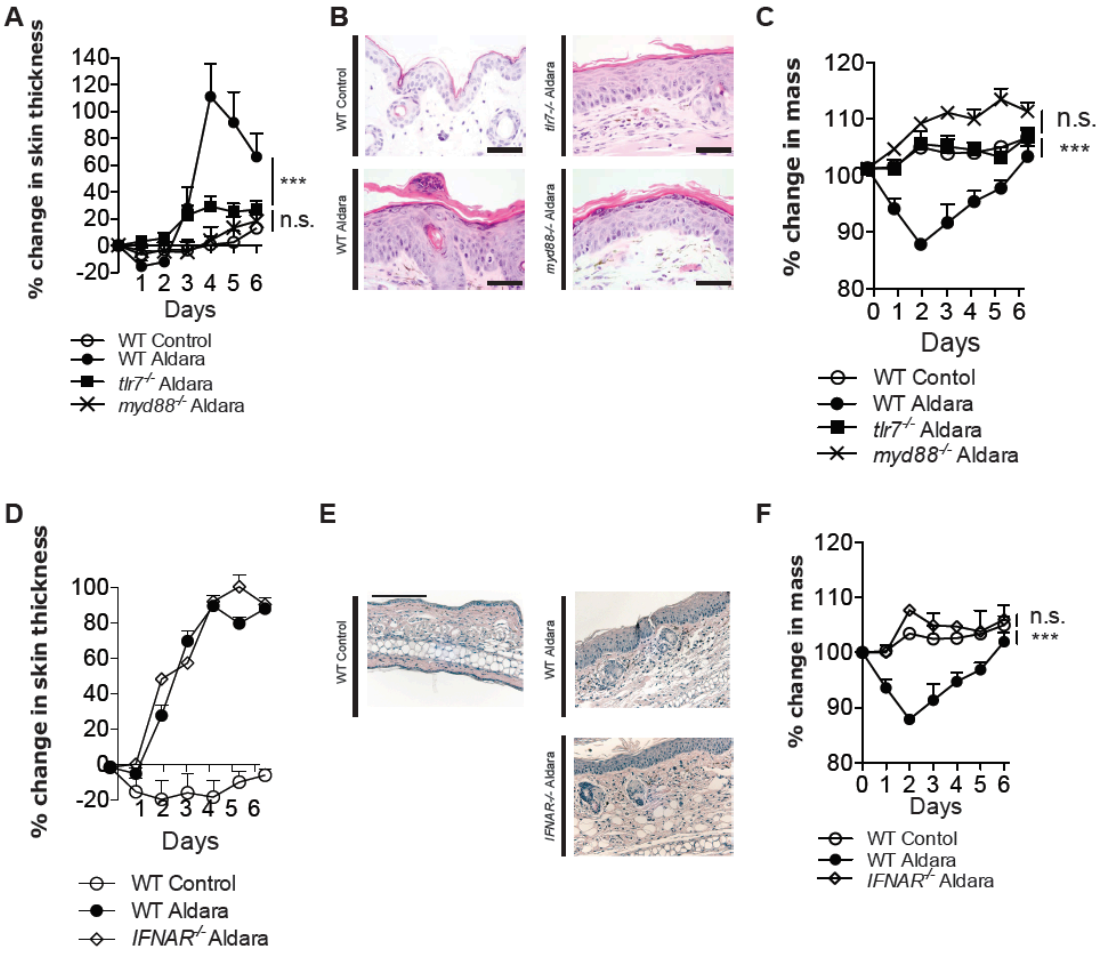

Figure 9. The signaling pathways involved in Aldara psoriasis (**A-C**) Comparison of WT versus tlr7$^{-/-}$ and *myd88*$^{-/-}$ mice (**A**) Kinetics of Aldara-induced skin inflammation (**B**) H&E staining. Original magnification: x40 Scale bar: 100 µm (**C**) Kinetics Aldara induced change in mass. (**D-F**) Comparison of WT and *IFNAR*$^{-/-}$ mice. (**D**) Kinetics of Aldara-induced skin inflammation (**E**) H&E staining. Original magnification: x40 Scale bar: 200 µm (**F**) Kinetics of mass change.

2.3 The roles of IL-17A, IL-17F and IL-22 in Aldara psoriasis

Previous report have shown upregulation of IL-17A, IL-17F and IL-22 mRNA in the skin of the Aldara treated mice after 72 hours[385]. This is in line with the findings in human psoriasis patients that have increased numbers of IL-17 and IL-22 producing cells in circulation[86]. In this model it is also supported by the fact that mice lacking IL-23 were resistant to Aldara[385], as well as successful treatment of Aldara psoriasis with anti-IL-12/23p40 antibody and implicates IL-23/T_H17 axis.

2.3.1 IL-17A, IL-17F and IL-22 are produced in the inflamed skin

The verification if the upregulation of the mRNA yielded increases in IL-17A, IL-17F and IL-22 on protein level was required. The skin lymphocytes from the Aldara treated and control mice were restimulated with PMA and ionomycin and stained intracellularly for IL-17A, IL-17F and IL-22. IL-17A and IL-17F producing cells were very abundant in the skin of Aldara treated animals, but not in controls. Interestingly, IL-17F production was about two-fold higher than that of IL-17A. There was also some production of IL-22, which also was only observed in Aldara treated animals (Figure 10A).

2.3.2 IL-17A, IL-17F and IL-22 play differential roles in the Aldara psoriasis

Earlier findings showed that IL-17RA deficient mice are resistant to Aldara psoriasis[385]. However, it is known that IL-17RA is not only the receptor for IL-17A and IL-17F, but also for IL-17C and IL-17E[256]. To check the differential roles of IL-17A, IL-17F and IL-22, single cytokine knockout animals were treated with Aldara and compared to wild-type counterparts and controls. Significant reductions in psoriasiform phenotype were observed in all of the mutant mouse strains, compared to wild-type mice. Analysis of skin thickness at peak disease (day 4) underlined the extent of the differences in lesion development (Figure 10B). Moreover, histological analysis of the inflammation revealed significant reductions in acanthosis in *Il17a*$^{-/-}$, *Il17f*$^{-/-}$, *Il22*$^{-/-}$ mice compared to wild-type mice, with the most pronounced effects observed in mice lacking IL-17F and IL-22 (Figure 10C).

2.3.3 IL-17AF heterodimers are more abundant in Aldara treated mice

The fact that there were more skin-invading cells secreting IL-17F than IL-17A is in

line with the stronger disease resistance in *Il17f*$^{-/-}$ compared with *Il17a*$^{-/-}$ mice. IL-17AF heterodimers have been described to share the biological properties with IL-17A and IL-17F[438].

This could explain the differential phenotype in mice lacking IL-17A vs. IL-17F. To verify if IL-17AF heterodimers are abundant after Aldara treatment a bead microarray analysis of the supernatants from restimulated lymph node and ear lymphocytes was performed. Indeed, the abundance of IL-17AF heterodimers was significantly increased in the lymph nodes of Aldara-treated mice, compared to controls. At the same time, no significant difference was observed in the ears (Figure 10D).

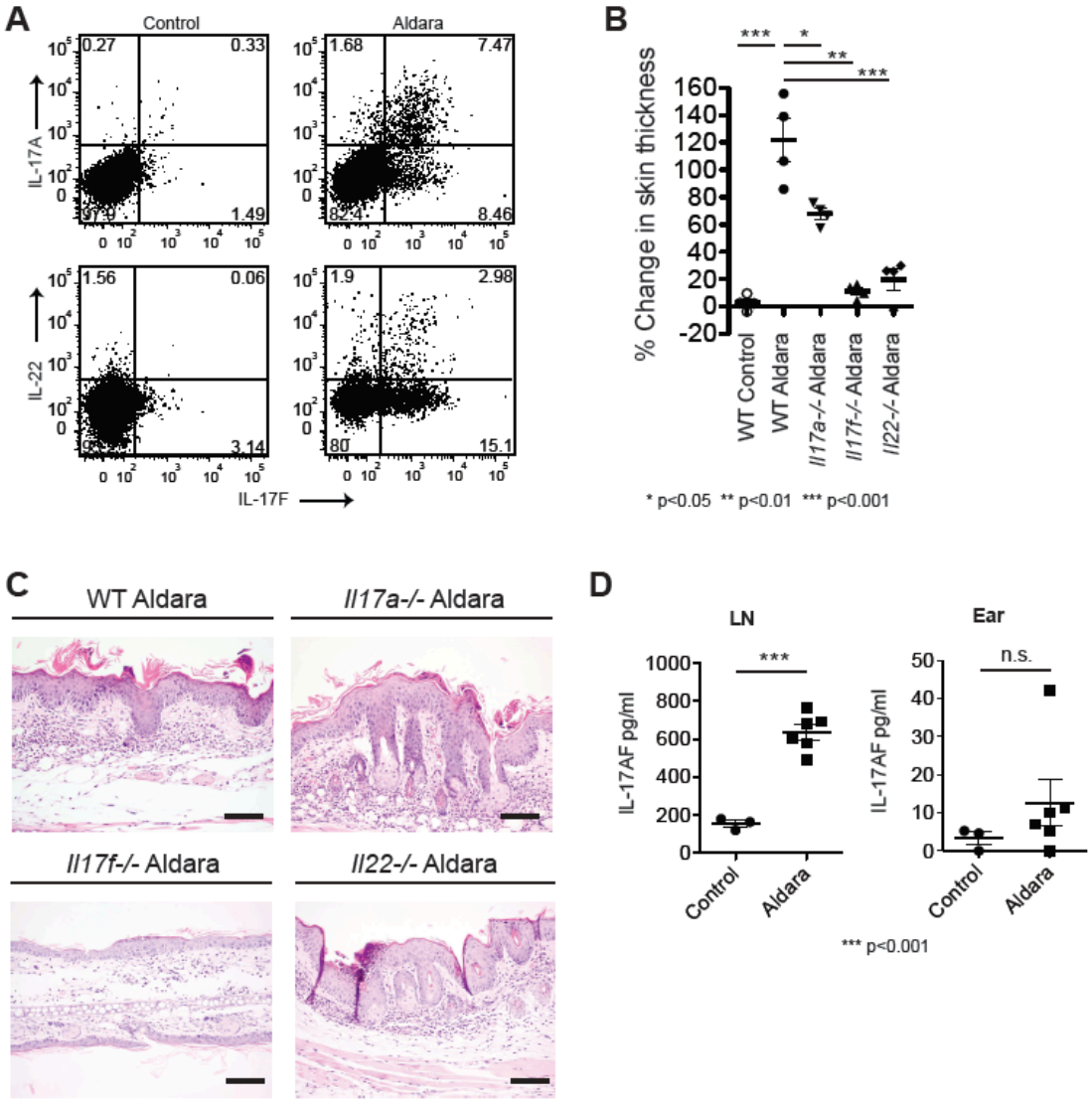

Figure 10. The roles of IL-17A, IL-17F and IL-22 in Aldara psoriasis. (**A**) Dot plots display the secretion of IL-17A, IL-17F, and IL-22 among CD45^{+} cells from the skin of wild-type mice on day 5 of Aldara treatment. (**B** and **C**) WT, *Il17a*$^{-/-}$, *Il17f*$^{-/-}$, and *Il22*$^{-/-}$ mice were treated with Aldara or control cream for 5 days. Scatter plot shows percent increase in skin thickness ($n = 4$) (**B**). Skin sections of Aldara-treated mice taken on day 6 (**C**) were stained with H&E. Scale bar: 100 µm. (**D**) IL-17AF heterodimer concentration was measured in the supernatant of LN or skin cells cultured for 24 hours using IL-17AF FlowCytomix Simplex kit.

2.4 The cellular sources of IL-17 and IL-22

Thus far the production of IL-17A, IL-17F, and IL-22 seemed to be crucial for the psoriasiform plaque formation in Aldara treatment model. It is also in line with the paradigm of the T_H17 cells[242], which were reported to produce the above effector cytokines in this model[385]. However, due to the rapid disease development other and especially innate sources of IL-17 and IL-22[386] could be involved in Aldara psoriasis.

To determine the main producers of these critical cytokines, Rorc-Cre x EYFP fate-mapping mouse strain[439] was used. In these mice EYFP gene flanked by loxP sites is inserted into ROSA26 locus. At the same time Cre recombinase is expressed under the *Rorc* promoter. Therefore, any cell that had at any point during its life cycle expressed *Rorc*, would be EYFP positive (Figure 11)[440]. *Rorc* is the gene for transcription factor Rorγt, which is the main transcription factor for IL-17A and IL-17F[209]. Even though no putative binding sites for Rorγt were found on the promoter region of the *il22* gene, it has been reported that mice and cells lacking *Rorc* do not produce IL-22[441]. Therefore, the vast majority of IL-17 and IL-22 producing cells in Rorc-Cre x EYFP mice would also be EYFP positive.

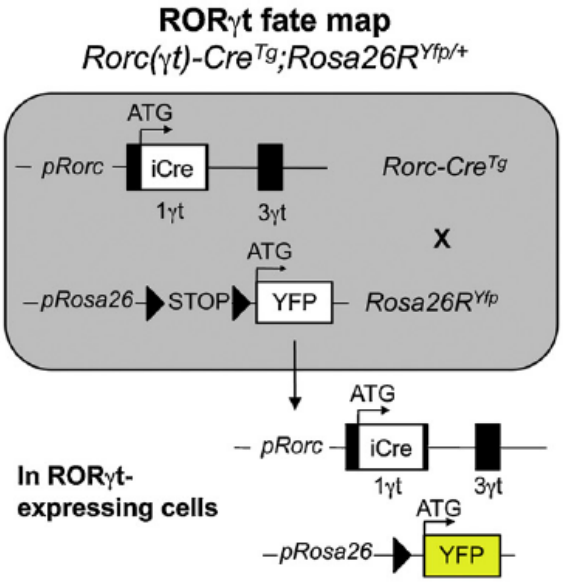

Figure 11. The mechanism behind Rorc-Cre x EYFP fate-mapping mouse. "RORγt-fate map mice" (Rorc(γt)-CreTg;Rosa26REyfp/+) allow the detection of cells that have expressed RORγt at any time of their lineage commitment ($EYFP^+$). Adapted from Vonarbourg and Diefenbach 2012.

2.4.1 γδ T cells are the main producers of IL-17 and IL-22 in Aldara psoriasis

To determine the cell types that are responsible for the production of IL-17 and IL-22

in the skin of Aldara treated mice the lymphocytes from the skin of these mice were restimulated and intracellularly stained for IL-17 and IL-22. Subsequently, to determine the main producers of these cytokines gating was performed on cytokine and EYFP double positive cells. As expected, the primary source of these cytokines were $CD3^+$ T cells (Data not shown). Interestingly, only a very small proportion of the cytokine producers were $CD4^+$ cells. However, around 80% of IL-17A and approximately 50% of IL-22 were produced by γδ T cells (Figures 12A and B).

2.4.2 γδ T cells are increased in the skin of Aldara treated mice

Thus far it was determined that γδ T cells are the main producers of the effector cytokines in Aldara psoriasis. Therefore these cells needed to be characterised in greater detail. The murine epidermis is populated by a unique population of γδ T cells, which are also called dendritic epidermal T cells (DETCs). These cells have been implicated in wound healing of the skin[442], but apart from one study[412] have not been reported to produce IL-17. At the same time it was recently reported that murine dermal resident γδ T cells express Rorγt and are potent producers of IL-17[418,419].

To understand the roles of each subset during the development of Aldara psoriasis γδ T cells in the Aldara treated vs. control skin were analysed. The DETCs are $V\gamma5^+$ and it was shown that the primary population of dermal resident γδ T cells are $V\gamma4^+$[419]. The analysis revealed two distinct populations of $TCR\gamma\delta^+$ cells in the skin. Interestingly, the $TCR\gamma\delta^{high}$ cells remained constant, while $TCR\gamma\delta^{medium}$ cells were substantially increased in the skin of Aldara treated mice (Figure 12C). As expected, $TCR\gamma\delta^{high}$ cells were found to be exclusively $V\gamma5^+$ DETCs, while $TCR\gamma\delta^{medium}$ were represented primarily by $V\gamma4^+$ cells (Figure 12D). After analysing the two γδ T cell populations in greater detail it was determined that the percentage and the absolute numbers of DETCs remained relatively constant between Aldara treated and control skin. At the same time the $V\gamma4^+$ proportion and the absolute numbers were significantly increased in the skin of Aldara compared to control treated mice (Figures 12E and F).

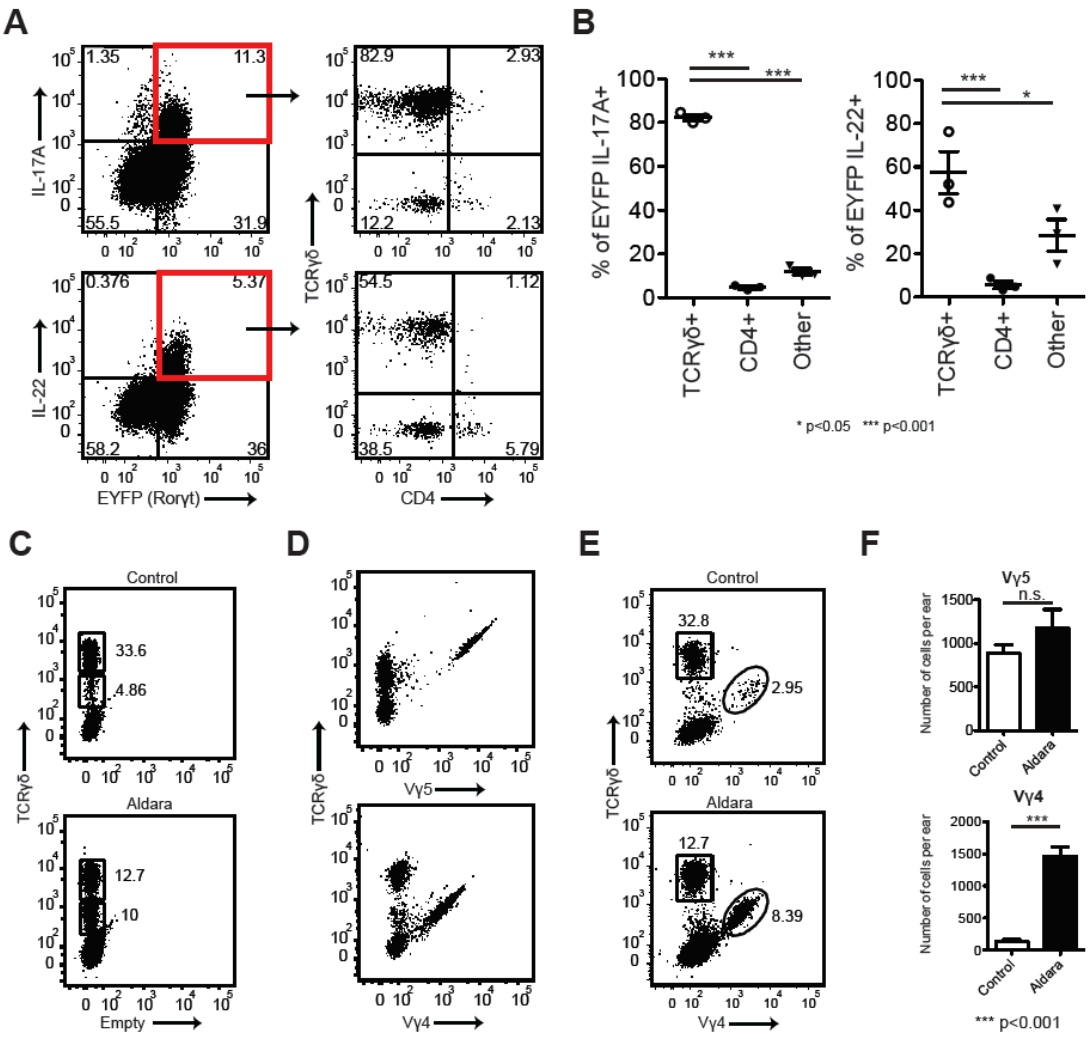

Figure 12. RORγt$^+$ γδ T cells are the main producers of IL-17A, IL-17F, and IL-22 in psoriasiform plaques. (**A**) Intracellular cytokine staining in the skin of Rorc-Cre × EYFP mice after 5 days of Aldara treatment, gated on CD45+ cells (n = 3), with (**B**) scatter plots showing percent distribution (n = 3). (**C**) Dot plots of different TCRγδ+ cell populations in the skin of WT mice treated with Aldara, analyzed on day 5 gated on CD3+ cells (n=4). (**D**) Dot plots of Vγ chain use by different γδ T cell populations in the skin of WT mice treated with Aldara, gated on CD3+ cells (n=3). (**E-F**) Plots display the percentages (**E**) and absolute cell number (**F**) of Vγ5+ and Vγ4+ cells among CD45+ cells isolated from the ear skin of WT mice treated with Aldara or control cream for 5 days (n=3).

2.5 γδ T cells are the main drivers of the Aldara psoriasis

So far the results are in line with the recent findings that γδ T cells are a potent source of IL-17 and IL-22 in models of autoimmunity[386,410,416]. However, the roles of T_H17 cells, $CD8^+$ and T_{regs} have not been excluded. Moreover, the exact roles of $V\gamma5^+$ DETCs vs. $V\gamma4^+$ have not been established. It is possible to differentially target αβ and γδ T cells in mice through knockouts of the TCR β and δ chains, respectively. But

there are a couple of drawbacks to this method. Firstly, *TCRb*$^{-/-}$ mice lack T_{reg} cells, together with the helper and the cytotoxic T cells. Secondly, these mice have higher abundance of γδ T cells. Finally, *TCRd*$^{-/-}$ mice lack all subsets of γδ T cells, thus making it impossible to study differential roles of Vγ5^{+} DETCs and Vγ4^{+} cells in Aldara psoriasis.

2.5.1 γδ T cells, but not T_H17 cells initiate Aldara psoriasis

To establish the relative roles of αβ and γδ T cells in Aldara psoriasis, the responses to Aldara were compared in *Tcrb*$^{-/-}$, *Tcrd*$^{-/-}$, and wild-type mice. *Tcrb*$^{-/-}$ mice developed inflammation similar to wild-type mice. In contrast, *Tcrd*$^{-/-}$ mice had drastically lower, but still noticeable inflammation in the skin (Figure 13A). Therefore, it is clear that γδ T cells are the main players in Aldara psoriasis. However, residual inflammation in *Tcrd*$^{-/-}$ mice suggests that another cell type can be involved in Aldara skin inflammation. Interestingly, there was also tendency for *Tcrb*$^{-/-}$ mice to develop slightly more inflammation than wild-type mice. This could be explained either by higher frequency of γδ T cells in those mice or the lack of regulatory T cells. It is also possible that the γδ T cell pool in *Tcrb*$^{-/-}$ mice is altered due to the developmental changes in the γδ lineage in the thymus[443]. However, Cai and colleagues, using *Tcra*$^{-/-}$ mice, also found them to be fully susceptible to IL-23–induced psoriasis-like plaque formation[444], indicating that the lack transconditioning of γδ T cells does not affect the phenotype of *Tcrb*$^{-/-}$ mice.

2.5.2 Vγ4^{+} γδ T cells, rather than DETCs produce IL-17 and IL-22

Due to some discrepancies in the field of γδ T cell research[412] and the experiments conducted up to this stage it can not be excluded that DETC also play a role in Aldara psoriasis. To further investigate which γδ T cell subset plays a more important role in this skin inflammation intracellular staining for IL-17 and IL-22 of the restimulated skin lymphocytes was performed. Gating was differentially conducted on Vγ5^{+} DETCs and Vγ4^{+} cells from the inflamed skin. Vγ4^{+} cells produced large amounts of IL-17 and IL-22, whereas DETCs did not (Figure 13B). This suggests that Vγ4^{+} cells drive the inflammatory skin alteration in this model.

2.5.3 DETCs do not play a role in Aldara psoriasis

Thus far the findings strongly point at the Vγ4$^+$ cells to be the main mediators of Aldara psoriasis. These cells were increased in the skin after Aldara treatment and were found to produce IL-17 and IL-22, which are necessary for the disease development. However, due to their the importance of DETCs in wound healing they can play a protective, rather than pathogenic role in this disease model[350]. To formally rule out the involvement of DETCs in Aldara psoriasis wild-type mice were compared to *Il15ra*$^{-/-}$. In the latter strain DETCs do not develop (Figure 13C) and therefore it would allow to establish the relative contribution of this cells type to the Aldara-driven inflammation. Surprisingly, there was no difference between the two mouse strains in response to Aldara treatment (Figure 13D). Therefore, at this point it was clearly established that Vγ4$^+$ γδ T cells are the main initiators of Aldara psoriasis.

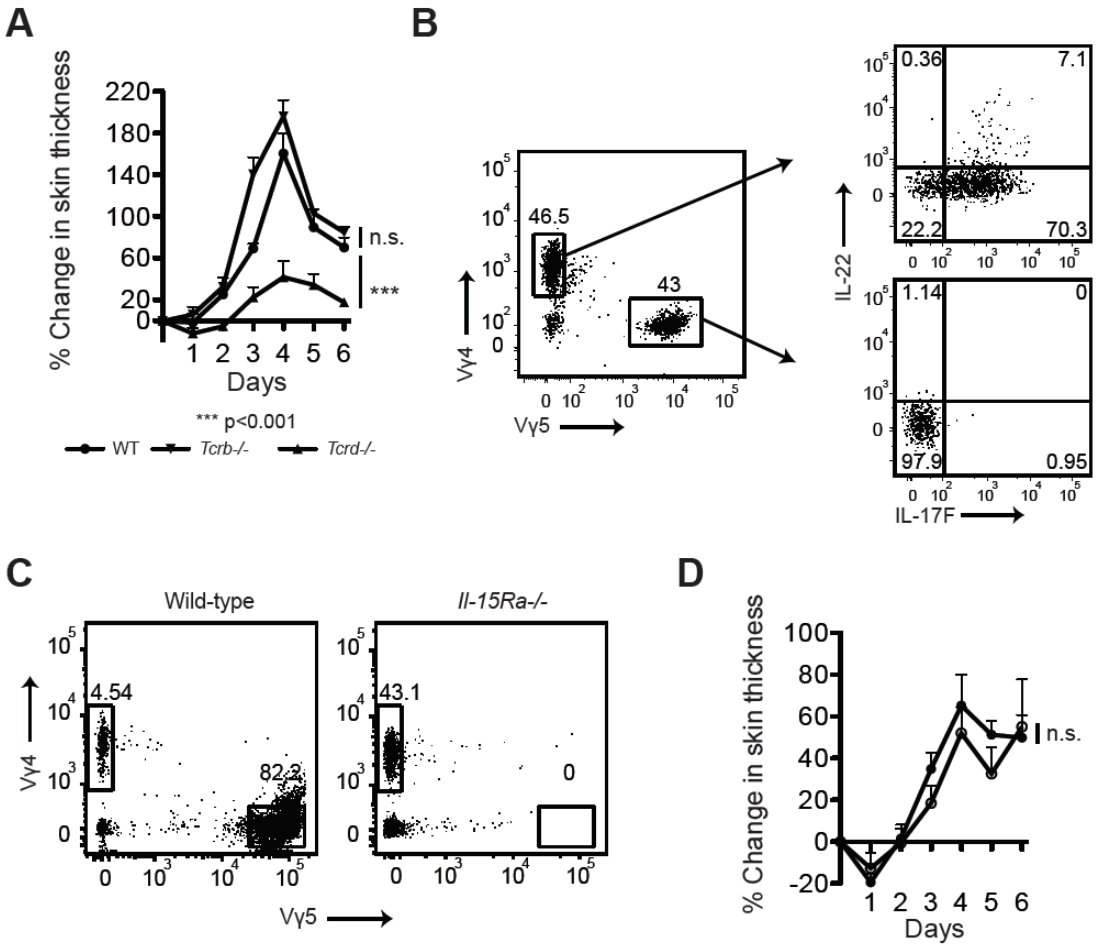

Figure 13. Vγ4$^+$ γδ T cells are the main initiators of Aldara psoriasis. (**A** and **D**) Kinetics of Aldara-induced skin inflammation in (**A**) WT versus *Tcrb*$^{-/-}$ and *Tcrd*$^{-/-}$ mice (n = 4) (**B**) Staining of V4γ$^+$ versus Vγ5$^+$ for IL-17F and IL-22, pre-gated on TCRγδ$^+$ cells. (**C**) Dot plots of Vγ4+ and Vγ5+ γδ T cell populations in the skin of naïve WT and *Il-15Ra*$^{-/-}$ mice gated on TCRγδ+ cells (n=3). (**D**) WT versus *Il15ra*$^{-/-}$ mice (n = 3).

2.6 Clonally expanded Vγ4$^+$δ4$^+$ γδ T cells infiltrate the skin

In previous studies it has been shown that in mice dermal rather than circulating γδ T cells produce IL-17 and initiated the immune response against *Mycobacterium tuberculosis*[419]. On the contrary, in human psoriasis γδ T cells were shown to infiltrate lesional skin through expression of skin homing receptors inclusing CCR4, CCR6, CCR10 and CLA. Moreover, these cells produced significant amounts of IL-17 and were normalised in the blood after successful treatments of patients for psoriasis[445]. In the study in mice it was shown that the dermal γδ T cells are radio resistant. Therefore, to address the question of dermal vs. circulating γδ T cell involvement in the immune response the bone marrow chimera approach was used. However, after initially also using this approach to determine the origin of the IL-17-producing γδ T cells were none found in the skin of Aldara treated mice (Data not shown). This finding has been recently explained by the study showing that IL-17-producing γδ T cells can only develop in the foetal thymus[446].

2.6.1 Vγ4$^+$ γδ T cells expand in the skin and in the lymph nodes

The second approach to address the question of the origin of IL-17-producing Vγ4$^+$ γδ T cells initiating Aldara psoriasis was to identify the site of their proliferation. This was done by staining the cells from the draining lymph nodes and the skin of Aldara treated and control mice for Ki-67, which is only expressed by dividing cells[447]. However, the analysis of Ki-67 staining revealed that Vγ4$^+$ γδ T cells expand both in the draining lymph nodes and in the skin of Aldara treated mice. Interestingly, CD4+ cells did not proliferate at either site, while DETCs seemed to have reduced proliferation after Aldara treatment (Figure 14A).

2.6.2 Vγ4$^+$ γδ T cells in the Aldara treated mice specifically upregulate CLA

Another approach to establish the origin of IL-17-producing Vγ4$^+$ γδ T cells in the Aldara treated skin used the expression of skin homing receptors on these cells. These include CCR4, CCR6, CCR10[448] and CLA[449] and have been shown to be expressed by γδ T cells in psoriasis patients[445].

Neither CCR4 nor CCR10 positive γδ T cells could be found in the skin and in the lymph nodes of Aldara or control mice. Interestingly, the vast majority of IL-17-producing CD27$^-$ γδ T cells were CCR6 positive, which also seems to be a marker for

the expression of Rorγt by the cells (Data not shown). The staining for CLA revealed that there were hardly any positive Vγ4$^+$ cells in control mice. But that CLA was specifically upregulated on CD27$^-$ Vγ4$^+$ cells in the draining lymph nodes of Aldara treated mice (Figure 14B and data not shown). This suggested that due to Aldara treatment the proliferating Vγ4$^+$ T cells are specifically recruited to the skin through induction of CLA and possibly through CCR6-CCL20 interaction[450].

2.6.3 Vγ4$^+$δ4$^+$ are the primary skin-invading γδ T cells population

There have been multiple reports of clonally expanded T cells found in psoriatic lesions[282]. At the same time, in the mouse model of collagen-induced arthritis it was shown that the primary source of IL-17 in the inflamed joints were clonally expanded Vγ4$^+$δ4$^+$ γδ T cells[416].

In depth analysis of Vγ4$^+$CD27$^-$CLA$^+$ γδ T cells in the draining lymph nodes of Aldara treated mice revealed that the skin-homing CLA$^+$ γδ T cells are indeed oligoclonal and similarly to the arthritis model were Vγ4$^+$δ4$^+$ (Figure 14C). Subsequent characterisation of the Vγ4$^+$ in the skin of Aldara treated mice has shown that a substantial proportion of skin infiltrating CLA$^+$ γδ T cells were also Vγ4$^+$δ4$^+$ (Figure 14D).

In summary, the results so far indicate that in the Aldara psoriasis model IL-17A, IL-17F and IL-22 are important for the disease development. The primary source of these cytokines were Vγ4$^+$ γδ T cells and not the T_H17 cells. Moreover, it seems that these cells are clonally expanded (Vγ4$^+$δ4$^+$) in the draining lymph nodes of Aldara treated mice and through upregulation of skin-homing molecule CLA are recruited to the skin where they cause the inflammation through production of effector cytokines IL-17 and IL-22. These findings are also very much in line with the clinical report, in which IL-17-producing γδ T cells were recruited to the psoriatic skin from circulation through expression of CLA and other skin homing receptors[445].

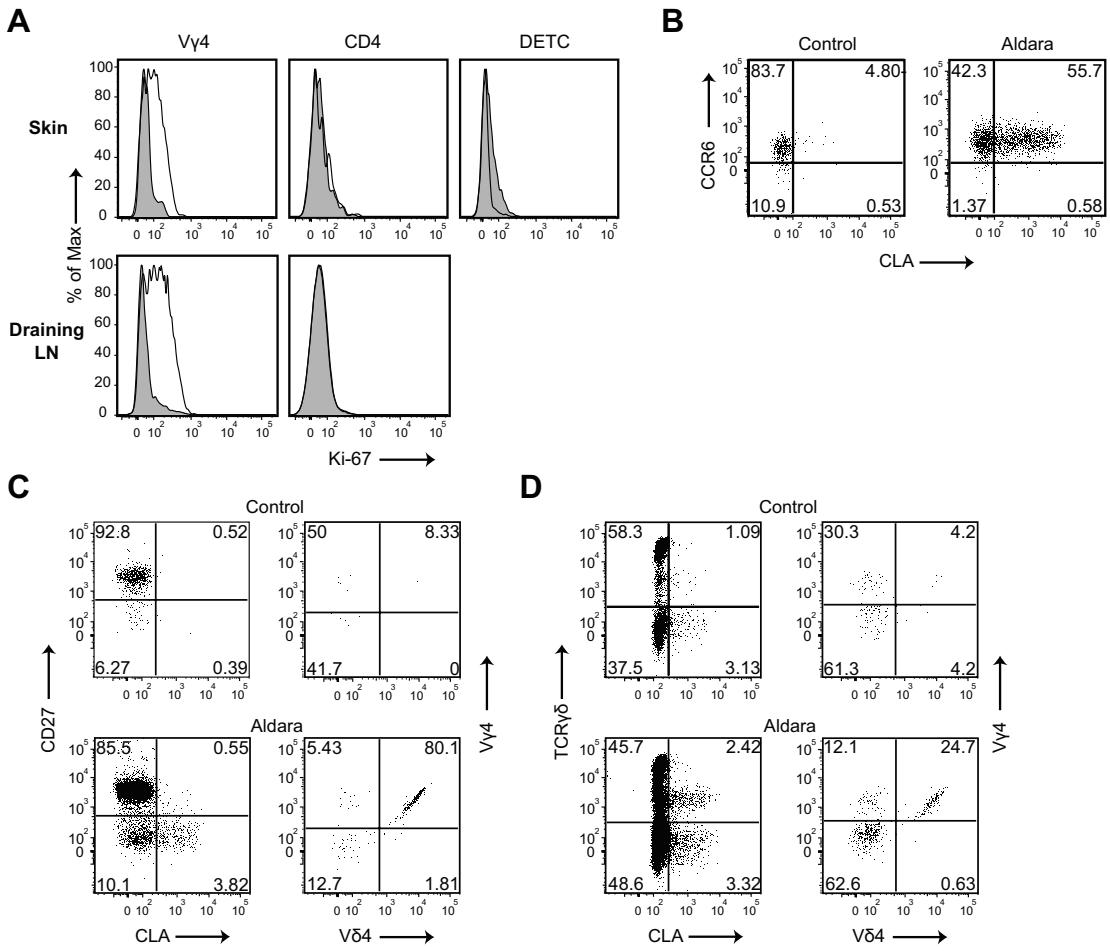

Figure 14. Clonally expanded Vγ4$^+$δ4$^+$ γδ T cells infiltrate the skin. (**A**) Vγ4$^+$, CD4$^+$ T cells and DETCs derived from the back skin and the draining lymph nodes of Control (shaded) vs. Aldara treated (transparent) mice on day 5 (pre-gated on CD45$^+$ live cells) were analyzed for the expression of Ki-67. (**B**) Flow cytometric analysis of CLA and CCR6 expression in the draining lymph nodes of Aldara treated and control mice on day 5, pre-gated on Vγ4+ cells. (**C-D**) Flow cytometric analysis of CLA$^+$ γδ T cells (**C**) in the draining lymph nodes pre-gated on γδ T cells, and (**D**) in the skin, pre-gated on CD45$^+$ cells.

2.7 ILCs are an alternative source of IL-22 in Aldara psoriasis

As previously described, innate lymphoid cells (ILCs) are a very minor population of lineage negative lymphocytes that has been shown to produce significant amounts of IL-22 and some IL-17 in certain disease models, especially in *Rag*$^{-/-}$ mice[428]. From Figures 12A and B it is clearly evident that γδ T cells are the primary producers of IL-17 and IL-22. And from Figure 13A it is the quite apparent that they are main cell type responsible for the development of Aldara psoriasis. However, it is also clear that there is an alternative source(s) of IL-17 and IL-22. Especially in case of IL-22 there seems to be another cell type that significantly contributes to Aldara-induced

psoriasis. Therefore, ILCs seem likely to be the likely source.

2.7.1 *Rag1*$^{-/-}$ mice have slightly stronger response to Aldara than *Tcrd*$^{-/-}$

To address the question of the involvement ILCs in Aldara psoriasis, wild-type mice were compared to *Tcrd*$^{-/-}$ and *Rag1*$^{-/-}$ counterparts. The latter mice lack all lymphocytes that use VDJ recombination including αβ and γδ T cells. Both strains were relatively protected from Aldara-induced skin inflammation. However, there was a tendency for the *Rag1*$^{-/-}$ mice to be slightly more inflamed than *Tcrd*$^{-/-}$ (Figure 15A). This finding implied the involvement of ILCs in Aldara psoriasis.

2.7.2 Lin^{-} cells produce IL-22 in the skin of Aldara treated mice

An unidentified population of CD3^{-} cells was previously found to produce substantial proportion of IL-22 in the skin of Aldara treated mice. As ILCs are Lin^{-} the lymphocytes from the skin of control and Aldara treated mice were stained for lineage markers CD3, CD11b, CD11c, B220 and Gr1. After restimulation these cells were also intracellularly stained for IL-22. Indeed, there was a small population of cells, which was negative for all of the lineage markers and that produced IL-22 only in the skin of Aldara treated mice. Interestingly, unlike the previous findings by our group[427], these cells were NKp46^{-} (Figure 15B).

2.7.3 ILCs are enriched in the spleens of *Rag1*$^{-/-}$ mice

Different groups use different markers to identify ILCs[425,428], yet the most reliable method seems to be a mixture of those and involves staining for CD3, CD5, CD11c, B220, Thy1, Sca-1 and IL-7Ra (Data not shown). This method involves taking CD3^{-} CD5^{-}CD11c^{-}B220^{-} cells in the first step of the gating to exclude the majority of lineage positive cells. As Thy1 is primarily a T cell marker[451] and Sca-1 is generally considered to be an antigen expressed by the stem cells[452] only a very small proportion of the remaining cells are positive for both of these markers. Finally, ILCs development is dependent on IL-7[422,424], therefore only IL-7Ra^{+} cells are considered to be *bona fide* ILCs (Figure 15C).

The above gating strategy was used to analyse the proportion of ILCs in the spleens of wild-type, *Tcrd*$^{-/-}$ and *Rag1*$^{-/-}$ mice. The overall proportion of the ILCs in the spleens was very low, and it was nearly the same for wild-type and *Tcrd*$^{-/-}$ mice. At the same

time, there was a significant increase in the proportion of these cells in the spleens of *Rag1*$^{-/-}$ mice, compared to the other two strains (Figure 15D). This finding is also in line with the hypothesis that ILCs are partially responsible for the induction of Aldara psoriasis through IL-22 production. Increased numbers of these cells in *Rag1*$^{-/-}$ mice could explain the tendency for slightly higher degree of inflammation in these mice compared to *Tcrd*$^{-/-}$ mice.

2.7.4 Increased upregulation of CLA on ILCs in *Rag1*$^{-/-}$ mice

γδ T cells were found to specifically upregulate CLA, which facilitated their migration to the skin in this psoriasis model and in patient studies[445]. The analysis of CLA expression on the ILCs in the draining lymph nodes of Aldara treated wild-type, *Tcrd*$^{-/-}$ and *Rag1*$^{-/-}$ mice revealed an increased upregulation of this skin homing marker in the latter strain (Figures 15E and F). Again, this further supports the role of ILCs in the development of Aldara psoriasis. It indicates that higher numbers of ILCs upregulate CLA to a higher degree to compensate for the absence of γδ T cells.

2.7.5 Increased production of IL-22 by ILCs in the skin of *Rag1*$^{-/-}$ mice

To aid the identification of ILCs the previously described Rorc-Cre x EYFP fate-mapping mouse strain was crossed onto the *Rag1*$^{-/-}$ background. In this mouse only ILCs would be positive of EYFP. This fact was used to compare the ILCs in the skin of wild-type and *Rag1*$^{-/-}$ mice after Aldara treatment in an unbiased way. The direct comparison of the two fate-mapped strains not only showed an increased proportion of ILCs in the skin of *Rag1*$^{-/-}$ mice, but also revealed an increased production of IL-22 by ILCs in *Rag1*$^{-/-}$ mice compared to wild-type counterparts (Figure 15G).

2.8 RORγt^{+} innate lymphocytes are essential for Aldara psoriasis

So far there is strong evidence that ILCs mediate Aldara psoriasis inflammation in the absence of γδ T cells. This hypothesis is supported by increased numbers of ILCs in *Rag1*$^{-/-}$ mice, their increased upregulation of CLA and production of IL-22 compared to wild-type and *Tcrd*$^{-/-}$ mice.

2.8.1 *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice are resistant to Aldara psoriasis

Rag2$^{-/-}$ *Il2rg*$^{-/-}$ mice do not have ILCs due to their lack of IL-7 receptor signalling. At

the same time these mice also do not have NK cells. However, their absence was accounted for in the *Il15Ra*$^{-/-}$ experiment, which showed no difference in the disease severity compared to wild-type mice. To have a direct comparison for the role of ILCs in the skin inflammation wild-type, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice were treated with Aldara. Astonishingly, *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice were completely resistant to Aldara-induced skin inflammation (Figure 16A), which underlinined the role of the ILCs in the Aldara psoriasis initiation.

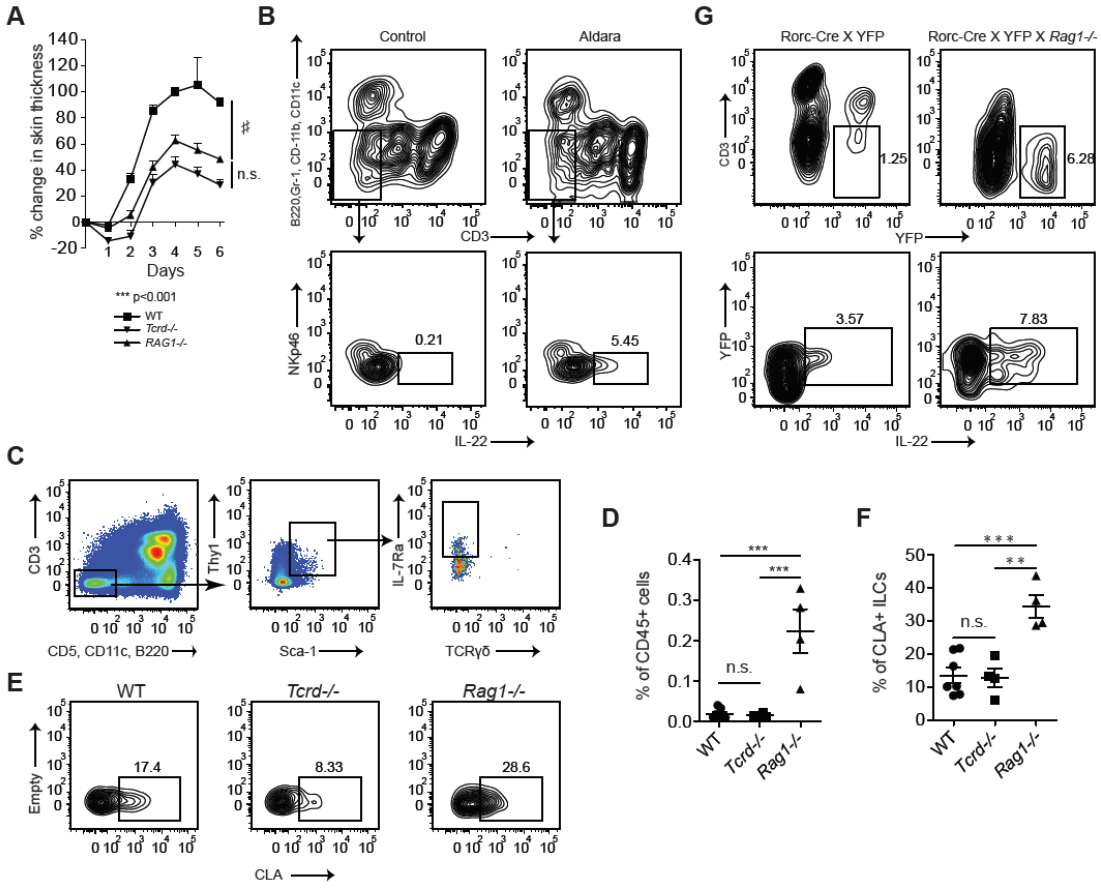

Figure 15. ILCs are the alternative source of IL-22 in Aldara psoriasis. (**A**) Kinetics of Aldara-induced skin inflammation in WT versus *Tcrd*$^{-/-}$ versus *Rag1*$^{-/-}$ mice. (**B**) Cytokine staining in ILCs in the skin of Aldara- versus control-treated mice, pre-gated on Lin^{-}CD45^{+} cells (n=3). (**C**) Gating strategy for ILCs, pre-gated on CD45^{+} cells (**D**) Scatter plots showing ILCs as a percentage of CD45^{+} cells (**E** and **F**) Analysis of CLA expression in WT vs. *Tcrd*$^{-/-}$ and *Rag1*$^{-/-}$ mice. (**E**) Representative FACS plots. (**F**) Scatter plot analysis. (**G**) Fate map analysis and IL-22 staining in the Aldara-treated skin of Rorc-Cre X EYFP and Rorc-Cre × EYFP × *Rag1*$^{-/-}$ mice gated on CD45^{+} cells.

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2.8.2 *Rorc*$^{-/-}$ mice are completely resistant to Aldara psoriasis

Thus far the results showed the dependence of Aldara psoriasis on ILCs and γδ T

cells. Both of these cell types are largely dependent on the transcription factor RORγt for the cytokine production of IL-17 and IL-22, which are crucial for the disease development. In line with this *Rorc*$^{-/-}$ mice were completely resistant to Aldara psoriasis and the changes in skin thickness in these mice were very similar to that of wild-type mice treated with the control cream (Figure 16B).

As previously stated mice lacking transcription factor RORγt do not produce IL-17 or IL-22[209,441]. As a result, no production of either cytokine was observed in Aldara treated *Rorc*$^{-/-}$ mice. This fact is especially underlined by slightly higher levels of IL-17 produced in the skin of control treated wild-type mice compared to *Rorc*$^{-/-}$ mice (Figure 16C). Taken together these findings reemphasize the importance of RORγt^{+} γδ T cells and ILCs, as well as their production of IL-17 and IL-22 for the development of skin inflammation in Aldara psoriasis model.

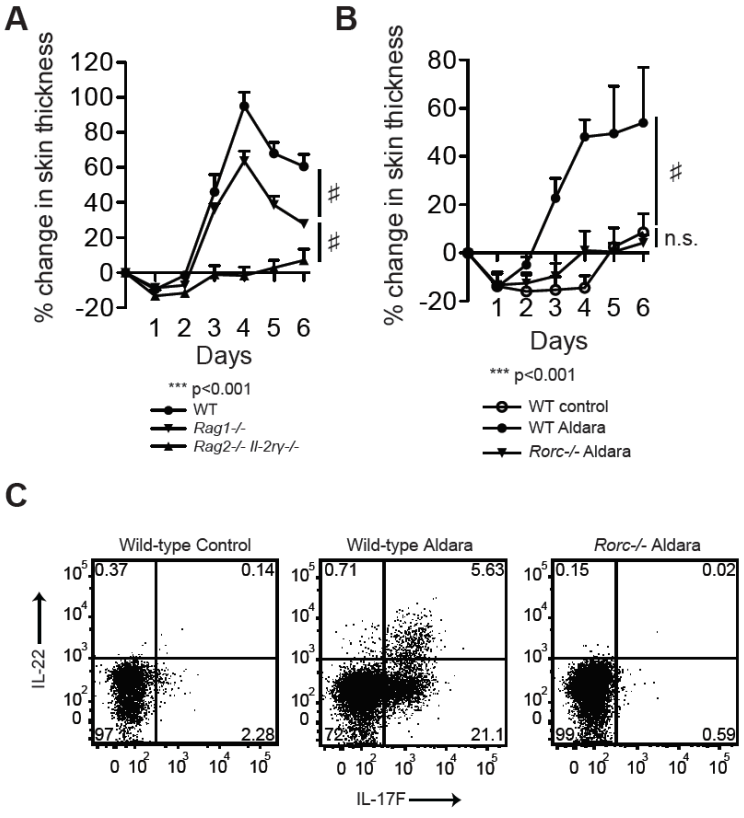

Figure 16. RORγt+ innate lymphocytes are essential for psoriasiform plaque formation. (**A**) WT versus *Rag1*$^{-/-}$ versus *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice and (**B**) WT versus *Rorc*$^{-/-}$ mice showing percentage change in skin thickness (n=3). (**C**) Staining for IL-17F and IL-22 in CD45^{+} cells from the skin of control and Aldara-treated WT versus *Rorc*$^{-/-}$ mice (*n* = 3)

3 Discussion

3.1 The model

Despite not being as authentic as the xenotransplantation model of psoriasis the Aldara model[385] and the cytokine injection models[377,380,381] present new promising approaches to study psoriasis. The big advantage of the Aldara model is that it is known to initiate psoriasis in humans[170-174]. Therefore one is tempted to speculate that unlike, the "shortcutting" method of cytokine injection, Aldara cream provides a *bona fide* psoriasis trigger. Moreover, previous[385] and this studies have shown quite conclusively that the Aldara model satisfies all of the criteria for a good psoriasis model, including psoriasis model[203], including: epidermal changes based on keratinocyte hyperproliferation and altered differentiation; papillomatosis; presence of inflammatory cells, including T cells, DC, and neutrophils; a functional role for T cells; and altered vascularisation[385]. It was also reported that Aldara psoriasis is reduced after NB-UVB treatment[433]. And in this study it was clearly shown that it also responds to anti-psoriatic drugs[453], namely anti-IL-12/23p40.

With the exception of streptococcal infections[43], Aldara is one of the more verified triggers of psoriasis to date. The studies of the former have been quite successful and yielded an identity of a potential antigenic determinant recognized by the T cells in the psoriatic skin. Namely, it was attributed to the cross-reactivity between streptococcal M protein and Keratin 17[282]. Despite this, streptococcal studies are unlikely to be translated to a mouse model, which makes the Aldara model[385] the one with the most authentic psoriasis trigger, with the exception of the xenotransplantation model.

The spontaneous disease models of psoriasis have been shown to be quite unauthentic due to lack of their T cells dependence and poor responsiveness to anti-psoriatic drugs. On the other hand, the transgenic models are useful for studying certain pathways in isolation with some of these models achieving great authenticity and resemblance of psoriasis[205]. However, psoriasis is known to have a complex pathogenic mechanism, which is still not fully understood. Therefore, despite their usefulness, the above models, similar to cytokine injection models generally target an already known pro-psoriatic cytokine or growth factor to initiate the disease, rather than study the actual trigger that resulted in the upregulation of a cytokine or a growth

factor in the first place

In this study and in others (Maries van den Broek personal communication) it has been shown that the pathogenic activity of Aldara is not solely driven by the agonistic activity of Imiquimod. This small molecule agonist of TLR7 has multiple activities itself, including triggering of TLR7, interaction with adenosine receptor signaling and direct apoptotic activity[431]. This has obvious advantages in that Aldara mimics a complex initial trigger for psoriasis. On the other hand, there are also disadvantages to this. Namely, it would be beneficial to delineate the initial trigger to a single stimulus rather than have at least four known activities for Aldara. Secondly, and more importantly not every person that is treated with Aldara succumbs to psoriasis, which is probably down to individual genetics.

The xenotransplantation model of psoriasis still remains the gold standard of psoriasis models with a lot of potential and some great discoveries that can be attributed to it[374]. However, this model also has its limitations. Namely, the difficulties with obtaining human skin grafts, a cumbersome and labour intensive experimental procedure and the time it takes for the disease to develop. It also became quite apparent that some of the findings in the xenotransplantation model, such as blocking IFN-α have failed to be translated into human anti-psoriatic treatments[376]. This model is said to be useful for studying developed psoriasis, when lesional skin is transplanted or the initiation of psoriasis if near-lesional skin is transplanted. However, a closer at the experimental settings of the psoriasis development model suggests clonal expansion of the pathogenic T cells that have been transplanted with the skin being modeled, rather than genuine initiation of psoriasis. Despite Aldara mouse model currently being used as psoriasis initiation model it also probably has potential for studying relapses of the disease and its exacerbation, as these activities have been reported in patient studies[170,171].

As shown above there are obvious limitations to mouse models in general, especially looking at the differences between the human and the mouse skin (Figure 6)[204,205]. At the same time, the immune systems are also quite different[161], making it difficult to directly translate the findings in the Aldara model into humans. This could be potentially circumvented by for example using humanized mice[454]. In light of this, the Aldara model may have good potential as a preclinical model for potential anti-psoriatic drug testing.

Even though psoriasis is a single disease there is quite a heterogeneity of psoriatic

phenotypes in patients (Figure 1)[20], which would implicate slightly different mechanisms involved in their development or different genetic backgrounds of the sufferers. The bimodal distribution of the disease with relatively different symptoms complicates modeling it and makes the studies of the disease even more difficult. At the same time, there is no universal cure for psoriasis with the most efficacious therapies not achieving more than 75% of responsiveness in patients[343]. The Aldara psoriasis is responsive to those known anti-psoriatic therapies. Therefore, it probably is tempting to speculate that it mimics the disease of the 75% of the responders. Thus, studies of this model could potentially provide nearly complete understanding of the disease in those individuals. With the age of personalized medicine quickly approaching this could indeed be very useful.

3.2 γδ T cells are central for Aldara psoriasis development

γδ T cells are a relatively novel subset of T cells, which acts at the interface between innate and adaptive immunity[455]. Even though some of the subsets of these cells have restricted T-cell receptors their ligands and consequences of the TCR signaling are still not well studied[456-458]. At the same time, it is known that these cells express a large variety of innate receptors including TLRs[409] and stress-sensors[459,460].

Psoriasis is thought to be caused by self-reactive or cross-reactive $CD4^+$ and/or $CD8^+$ cells[20,282]. It was previously briefly reported that γδ T cells are increased in the psoriatic lesions[420]. In this study of Aldara model these cells were clearly shown to play the central role in the development of skin inflammation. The mice lacking γδ T cells were highly protected from the disease. Interestingly, DETCs, which are thought to be important for wound repair[350] didn't seem to play a role as was shown in *Il15Ra*$^{-/-}$ mice.

Similar to the human disease high production of IL-17A, IL-17F and IL-22[86] was observed in the lesional skin of Aldara treated mice. Subsequently, all these cytokines were shown to play a role in the disease initiation, but with quite different outcomes. Interestingly, mice lacking IL-17F showed much reduced skin inflammation compared to IL-17A mice. There are some possible explanations for this. It was shown the levels of IL-17AF heterodimer were increased in the Aldara treated mice compared to the controls. At the same time, the production of IL-17F two-fold higher than IL-17A. Taken together, these data indicate that the majority of

the disease would be mediated by IL-17F homodimers or IL-17AF heterodimers. Hence, in the absence of IL-17A only the heterodimers would be affected, while the lack of IL-17F would result in the absence of both. Therefore, much less inflammation. Interestingly, it was recently shown that IL-17C seems to play a role in Aldara psoriasis[260], which could explain nearly complete resistance of IL-17RA deficient mice to the disease[385], but some residual inflammation in IL-17A and even IL-17F deficient animals.

The finding that γδ T cells produce IL-22 is a relatively rare one and the production of this cytokine was exclusive to the skin, but not the draining lymph nodes of the mice that have been treated with Aldara (data not shown). This implies that some extra signaling occurs in the skin, but not in the lymph nodes of the inflamed mice, enabling the production of IL-22. Potentially, the production of IL-22 could be triggered through AhR. However, its ligands are not very well studied and therefore it would be quite a challenge to understand if the signal may be comes from Aldara or from the host cells. At the same time, it could involve both keratinocytes, which have been shown to produce a lot of IL-1 upon activation and activated DCs that would produce IL-23. Both of these cytokines are potent inducers of IL-17 and IL-22 in γδ T cells[409,410]. A recent study has shown that in the absence of IL-17RA the mice get a delayed onset of the skin inflammation. Moreover, in that study in line with the later onset of the inflammation IL-22 is produced by both CD4 and γδ T cells[461].

γδ T cells have been shown to be potent and rapid producers of IL-17 and IL-22 in response to IL-23 and IL-1[410], as well as after TLR stimulation[409] and recently through the TCR engagement[457]. Due to the short disease course it was not surprising that γδ T cells were the main source of IL-17 and IL-22 in the Aldara model. Moreover, it is quite in line with the previous observations that anti-CD3 antibody treated and *RAG*$^{-/-}$ *2rg*$^{-/-}$ mice had reduced skin inflammation[385]. Interestingly, the similar approach was used in the xenotransplantation model, where the disease was blocked by OKT3 T cell depleting antibody[374], which also targets γδ T cells[462]. And both studies have implicated CD4^{+} T cells in the disease development. Moreover, a study that slightly preceded ours was able to show that IL-17-producing γδ T cells are indeed pivotal for the development of Aldara psoriasis[444]. These findings were also duplicated and reinforced in the cytokine injection model[380,444]. Therefore, our results that show γδ T cells being important for the development of Aldara psoriasis are further reinforced.

One of the more difficult questions of this study was to identify the source of γδ T cells. This was particularly tough due to inability to use bone-marrow chimeras to study this phenomenon[418,446]. Interestingly, recent report actually used this method to show a role for IL-17-producing dermal-resident radioresistant γδ T cells in subcutaneous immunization[419]. However, in the light of the above findings that IL-17-producing γδ T cells cannot be reconstituted with bone marrow[446] would make the dermal resident γδ T cells the only remaining IL-17-producing γδ T cells in those mice.

It was recently reported that circulating IL-17-producing γδ T cells were reduced in the blood of psoriasis patients and that these cells were rapidly recruited to the perturbed skin through upregulation of skin-homing receptors, including CLA. Moreover, the levels of these cells in the blood were restored after successful treatment of psoriasis in those patients[445]. Using a similar approach we were able to show that γδ T cells specifically proliferate in the Aldara treated mice and that this could take place both in the skin and in the draining lymph nodes. At the same time, skin homing molecule CLA was also specifically upregulated in the draining lymph nodes of Aldara treated mice. Moreover, CLA^+ γδ T cells were also found in the lesional skin of Aldara treated mice. These findings are quite in line with the patient studies and therefore indicate that IL-17-producing γδ T cells can be recruited to the stressed skin and potentially initiate a disease like psoriasis.

In the mouse model of collagen-induced arthritis it was found that the main source of IL-17 were oligoclonal γδ T cells. Those were responsible for the exacerbation of the disease[416]. In human psoriasis multiple reports have shown oligoclonality of T cells in psoriatic lesions[282]. Interestingly, in our study CLA^+ γδ T cells in the draining lymph nodes were also nearly exclusively oligoclonal $V\gamma4^+\delta4^+$. At the same time, a population of CLA^+ γδ T cells in the lesional skin was also $V\gamma4^+\delta4^+$, suggesting some specific and most likely host derived antigen recognition by these cells. However, a follow up study of the arthritis model was able to show that it is the mycobacteria from Complete Freud's Adjuvant (CFA) that were important for the IL-17 production by these cells. This cytokine production was also dependent on TLR adaptor MyD88 and the route of administration, favouring subcutaneous or intradermal route. The subsequent conclusions suggested that it is the dermal γδ T cells, through their expression of TLRs recognize mycobacteria in the CFA and then migrate to the draining lymph nodes and expand in a clonal fashion[1]. Our model is quite similar in a

way that the stimulus is applied to the skin and could even fit the proposed model, especially due to our finding that MyD88 deficient animals were resistant to the Aldara psoriasis. However, it seems quite unlikely that the Aldara triggered γδ T cells would first migrate from the dermis to the draining lymph nodes, then clonally expand there, upregulate CLA and subsequently return to the skin to cause the inflammation. What seems more likely is that in both cases MyD88 is important for the dendritic cells that recognize CFA or Aldara and upon activation migrate to the draining lymph nodes and promote the expansion of $V\gamma4^{+}\delta4^{+}$ oligoclonal cells, as well as upregultion of CLA. This theory is further reinforced by our findings that MyD88 in Aldara model is only required in the conventional DCs (data not shown).

As mentioned above the Aldara model mimics the initiation of psoriasis, whereas in patient studies the plaques are more likely to be well developed and therefore different. This means that the Aldara model does not in particular contradict the findings of CD4 and CD8 cells in the established psoriatic plaques of patients, but rather complements it. This idea is also favoured by the findings that γδ T cells are rapidly recruited to the perturbed human skin[445] and are increased in psoriatic lesions[420,445]. Interestingly, there has been a report of TLR7 being expressed by human γδ T cells[463]. Additionally, these cells were shown to express TLR2[409]. Interestingly, this PRR have also been shown to recognize streptococcal M protein[464]. Together with the findings in mice that γδ T cells promote CD4[419,465] and CD8[414] responses, as well as their potential function as an APC in humans[466] leaves quite a bit of room for speculation about the exact mechanism and roles of γδ T cells in the Aldara psoriasis and the human disease initiation processes.

Overall, the findings in this model are also quite complementary to the clinical efficacy of targeting the IL-23/IL-17 pathway[345-347,467]. A closer look at evolution further supports the idea that IL-17 is an innate cytokine as ancestors of this gene-family is preserved among invertebrate species[392]. Therefore it is not unexpected that more innate-like γδ T cells would be the main source of this cytokine, rather than the conventional adaptive T_H17 cells. It remains unclear whether γδ T cells also play a part in the chronification and relapses of psoriasis. Broad neutralization of a specific cytokine can be effective, but to more specifically target the pathogenic entity in psoriasis and other diseases of epithelial barriers (such as Crohn's disease) and to develop curative therapeutic strategies, more preclinical work is needed to better understand the pathogenesis of epithelial-inflammation disorders.

3.3 Innate lymphoid cells

ILCs have been shown to be rapid producers of IL-17, but more of IL-22 in response to IL-23 in many mouse models that lack T and B lymphocytes[245,428,468]. The similar findings are true for our study where they were major contributors to γδ T cell IL-22 production. This model is also dependent on IL-23 as IL-23p19 deficient mice were resistant to the skin inflammation[385]. Moreover, the role of ILCs was further supported by the findings of another group that have shown the dependence of Aldara inflammation on an innate source of IL-22[469].

However, the exact nature of the role of ILCs still remains unclear. They are increased in numbers in *RAG*$^{-/-}$ mice and therefore seem to play an important role in those models. But are they really a significant and important contributors of cytokine production under normal conditions and during inflammation such as psoriasis? Or are the ILCs may be more rapid censors of external pathogenic threat and cytokine producers than γδ T cells? To answer these questions and many others specific targeting of these cells would be required. Unfortunately, current understanding of these cells and their receptors hardly allows that and thus more research into their development, cell-specific markers and transcription factors are required.

4 Conclusions

In psoriasis, the current notion that IL-23 induces T_H17 cells stems from the observation that activated T cells are a major part of the skin-infiltrating immune cells and are a known source of IL-17 and IL-22. Only recently have innate lymphocytes been acknowledged to be highly effective producers of these mediators. Our data show that Rorγt-dependent ILCs and γδ T cells are necessary and sufficient to drive Aldara psoriasis in mice through the collective delivery of IL-17A, IL-17F, and IL-22 to the skin. Without dismissing adaptive immune processes in the etiology of psoriasis, our study does establish the sufficiency of a dysregulated innate immune compartment for psoriatic plaque formation. Thus, our proposed paradigm of lesion development not only provides a new basis for understanding the therapeutic efficacy of new biological drugs to treat human psoriasis, but may also lead to more in depth research on γδ T cell and ILC involvement in the human disease (Summarized in Figure 17).

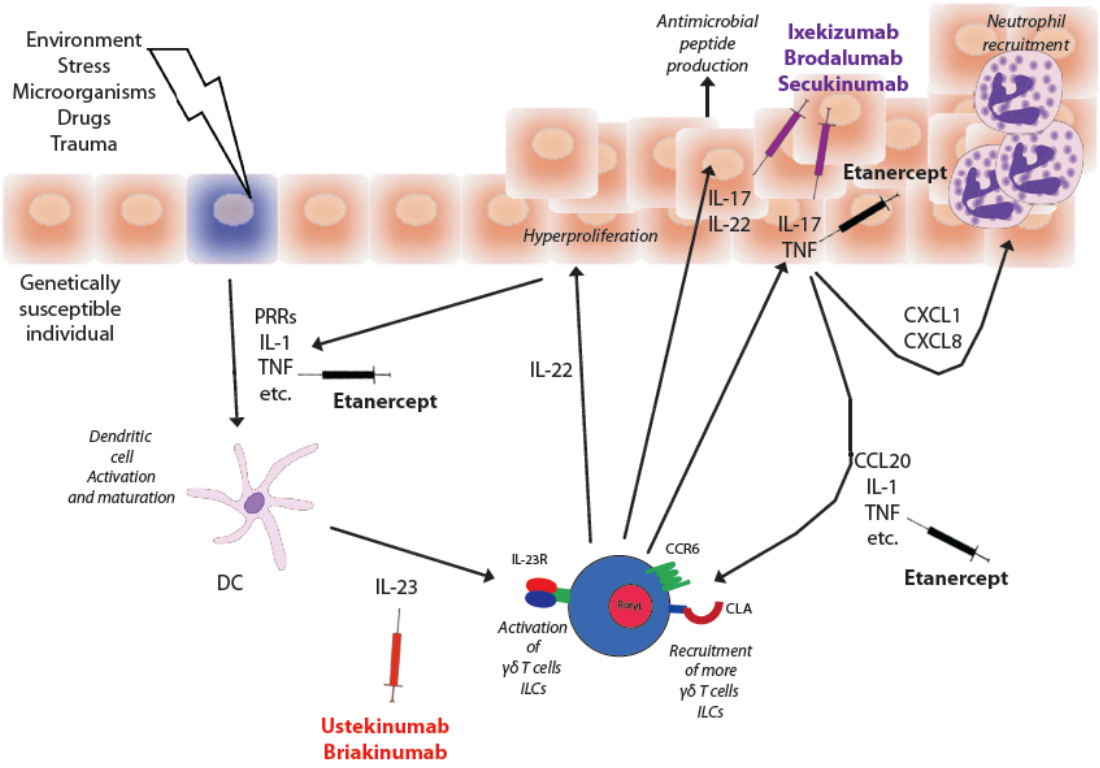

Figure 17. Initiation of psoriasis by γδ T cells and ILCs. Microbial insults, toxic substances other cellular stress lead either to direct activation of DCs through pattern recognition receptors (PRRs), athogen-associated molecular patterns (PAMPs) or indirectly through keratinocyte disturbances (IL-1 and TNF). Stress-sensing DCs produce IL-23, which activates γδ T cells and ILCs and though transcription factor Rorγt^{+} produce proinflammatory TNF, IL-17 and IL-22. IL-22 acts on keratinocytes inducing their proliferation and at the same time acts synergistically with IL-17 to promote production of antimicrobial peptides on keratinocytes. At the same time TNF and IL-17 trigger keratinocyte and more DC activation. This leads to yet more TNF production, upregulation of adhesion molecules (e.g. CD62E ligand for CLA), angiogenesis and chemokine production. These chemokines include CXCL1 and CXCL8, responsible for the recruitment of neutrophils, as well as CCL20, a CCR6 ligand expressed by γδ T cells and ILCs, resulting in a self-amplifying inflammatory loop. Syringes indicate current available anti-cytokine therapies for psoriasis.

4 Methods

4.1 *In vivo*

4.1.1 Animals

C57BL/6 mice were obtained from Janvier. *Rorc*$^{GFP/GFP}$ (in text referred to as Rorc$_{-/-}$), *Tcrβδ*$^{-/-}$, *Rag1*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Tcrb*$^{-/-}$ and *Tcrd*$^{-/-}$ mice were bred from *Tcrβδ*$^{-/-}$ mice. Rorc-Cre crossed with Rosa26-stop-eYFP mice (called Rorc-Cre × EYFP mice herein) were provided by A. Diefenbach (University of Freiburg, Freiburg, Germany), *Il17a*$^{-/-}$ mice by Y. Iwakura (University of Tokyo, Tokyo, Japan), *Il17f*$^{-/-}$ mice by Merck Serono S.A., *Il22*$^{-/-}$ mice by J.C. Renauld (Université Catholique de Louvain, Yvoir, Belgium), *Il15ra*$^{-/-}$ mice were provided by S. Bulfone-Paus (University of Giessen, Giessen, Germany). All animals were kept in house under specific pathogen–free conditions at a 12hour light/dark cycle with food and water provided *ad libitum*. All animal experiments were approved by the swiss cantonary veterinary office (33/2010).

4.1.2 Treatments

Briefly, The mouse back was shaved, and a daily dose of 50 mg of Aldara (5% IMQ cream; 3M Pharmaceuticals) or control vehicle cream (Soft Kreme KA, Kantonsapotheke Zürich) was applied on the back and 5 mg to each ear for 7 days unless otherwise indicated. Anti–IL-12/23p40 treatment was performed by i.p. injections of the mice with 200 µg/mouse rat anti–mouse IL-12/23p40 mAb (C17.8) and respective IgG2A isotype control antibody (2A3, both Bio X Cell) on day 2 of Aldara application.

4.1.3 Scoring

The skin and ear thickness were measured every day using caliper (Mitutoyo). The changes in skin and ear thickness were converted into percentage changes to make the differences between the experiments comparable to each other and plotted on scatter plots against days to show the disease course

4.2 *In vitro*

4.2.1 Cell isolation and preparation

Animals were euthanized with CO_2 and 4 cm^2 of the back skin and whole ears were collected. These were cut into small pieces with scissors and digested with 1 mg/ml collagenase type IA and 100 µg/ml DNase (Sigma-Aldrich) for 60 minutes at 37°C and 5% CO_2, followed by the addition of EDTA (final concentration 5mM) to stop the reaction. Isolation of leukocytes from spleens and the lymph nodes involved teasing them apart using frosted glass slides. Both were followed by filtering through 70-µm cell strainers (BD) and washed with PBS to obtain single-cell suspensions.

4.2.2 Surface staining for flow cytometry

To stain the cells single cell suspensions were incubated with antibodies for 20 minutes at 4°C in the dark. Antibodies used: B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD45 (30-F11), CD3 (17A2), CD4 (GK1.5), CD27 (LG.3A10), CLA (HECA-425), Gr-1 (RB6-8C5), CCR6 (29-2L17), NKp46 (29A1.4), TCRγδ (GL3), Vγ4 (UC10A6) and Vγ5 (536), (BD, eBioscience, R&D and Biolegend). The cells were washed twice with PBS and resuspended in 200 µl of PBS for acquisition.

4.2.3 Intracellular cytokine staining

For intracellular cytokine staining, cells were restimulated at 37°C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco or PAN Biotech). RPMI 1640 was supplemented with 10% FCS and Phorbol 12-myristate 13-acetate (50ng/ml) (Applichem), Ionomycin (500ng/µl) (Invitrogen) and Monensin (1µl/ml medium, GolgiStop™, BD) for restimulation and to block intracellular transport. After regular surface staining, cells were permeabilized using Cytofix/Cytoperm™ (BD) for 15 min. After washing the cells twice with diluted Perm/Wash™ (BD) buffer the cells were stained intracellularly for 20 minutes at 4°C in the dark. Antibodies used: IL-17A (TC11-18H10.1) (Biolegend) and IL-17F (eBio18F10) (eBioscience) and IL-22 (Genentech). After staining the cells were again washed twice with diluted Perm/Wash™ buffer and once with PBS and resuspended in 200 µl of PBS for acquisition.

4.2.4 Ki-67 staining

The cells were isolated as described above and pelleted. While vortexing, 5 ml of cold 70% - 80% ethanol was added drop by drop into the cell pellets. The cells were incubated at -20°C for 2 hours. The fixed cells were washed twice in 30 ml of wash buffer (PBS with 1% FBS, 0.09% NaN_3 pH7.2). The cells were stained with anti-Ki-67 antibody (20Raj1) (eBioscience) diluted 1:30 in PBS for 20 minutes at 4°C in the dark. Afterwards the cells were washed with PBS twice and resuspended in 200 µl of PBS for acquisition.

4.2.5 Preparation of samples for IL-17AF heterodimer bead array

The cells from ears and lymph nodes were restimulated at 37°C and 5% CO_2 in RPMI supplemented with 10% FCS and Phorbol 12-myristate 13-acetate (50ng/ml) (Applichem), Ionomycin (500ng/µl) (Invitrogen) and Monensin (1µl/ml medium, GolgiStop™, BD) for 5 hours to stimulate cytokine secretion. Afterwards the supernatants were collected for bead array.

4.2.6 Bead array IL-17AF heterodimer detection

The volumes of the reagents for the desired numbers of samples were calculated of reagent using FlowCytomix Pro Software (eBioscience). The lyophilized standard was reconstituted by adding distilled water according to the label on the standard vial to a final concentration of 40 ng/ml. The tube was swirled thoroughly to ensure quantitative solubilization of contents. 10 µl of reconstituted standard was added to a vial and filled up to the final volume of 200 µl with Assay Buffer (PBS with 1% BSA). 100 µl Assay Buffer was added to 6 tubes. 50 µl of standard was transferred from 1 to tube 2, mixing the contents of tube 2 and transferred 50 µl to tube 3. The procedure was repeated to create a row of 7 standard dilutions. The vial containing Allophycocyanin fluorescent anti-IL-17A monoclonal beads was vortexed for 5 seconds to resuspend the beads thoroughly. The beads were pipetted up and down and 1/20 of final volume was pipetted into a vial and filled up to the final volume with assay buffer. The beads were washed once. 1/20 of final volume of biotin conjugated anti-IL-17F polyclonal antibody was pipetted into a vial and filled up to the final volume with assay buffer. Streptavidin-Phycoerythrin (PE) was diluted 1:30 in assay

buffer.

All tests were performed in duplicates. 25 µl of Standard Mixture dilutions 1 to 7, assay buffer and samples were added to respective tubes. 25 µl of bead mixture and 50 µl of Biotin-anti-IL-17F were added to all tubes. The contents of the tubes were mixed thoroughly and incubated for 2 hours at room temperature. After the incubation the samples were washed twice with 1 ml of assay buffer. To detect the beads bound to IL-17AF heterodimers 50 µl of Streptavidin-PE was added to each tube and incubated at room temperature for 1 hour. The samples were again washed twice with assay buffer and resuspended in 200 µl of assay buffer for acquisition. The acquired data was analysed using FlowCytomix Pro Software.

4.2.7 Splenocyte cultures

Splenocytes from Rorc-Cre X YFP X *Rag1-/-* mice were cultured at 10^6/ml in complete RPMI, supplemented with DMSO (1:1000), Aldara (final concentration 2µg/ml), Imiquimod (Sequoia Research Products) (final concentration 0.1µg/ml), both initially dissolved in DMSO or DMSO (1:1000) and IL-23 (20ng/ml).

4.2.8 Flow cytometry and analysis

Beads and cells were acquired with FACS Canto II or FACS LSRII Fortessa (BD). Post-acquisition analysis of fluorescently stained cells was performed with FlowJo (Tree Star) software version 9.2.

4.2.9 Histology

For histology, animals were euthanized with CO_2, The skin or the ears were fixed in 4% Formalin, embedded in Paraffin and 3µm sections were processed for H&E staining Deparaffinized sections were stained using H&E. Immunohistological staining for MPO (1:100, rabbit, Thermo Scientific) was performed by a Ventana Benchmark XT automated staining system according to the manufacturer's guidelines

4.3 Statistical analysis

For For disease severity, differences between groups were evaluated by 2-way ANOVA with Bonferroni's post hoc test. For analysis of scatter plots of maximum

thickness comparing ≥3 groups of mice, 1-way ANOVA with Bonferroni's post-test was used. Differences between two sets of data were evaluated by 2-tailed Student's *t* test. Data represent mean ± SEM. $P \leq 0.05$ was considered statistically significant. All statistics were done using GraphPad Prism (GraphPad Software).

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Education:

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October 2007- October 2008	**Imperial College London** **Biochemical Research MRes** Projects: "Structural studies of N-terminal fragment of Latrophilin, a neuronal receptor of α-latrotoxin" "The Role of Bid in the mammalian cell cycle" "Increased surveillance of cells undergoing mitosis by human NK cells"	London, UK
October 2004- July 2007	**Imperial College London** **Biochemistry BSc,** Final Year Project: "Isolation of recombinant antibodies for endogenous Latrophilin receptor "LASSO" using phage display".	London, UK

Secondary education:

2002-2004	**St. Edward's School**	Oxford, UK
2000-2002	**King's College**	Taunton, UK

Publications:

Pantelyushin, S., Haak, S., Ingold, B., Kulig, P., Heppner, F.L., Navarini, A.A., and Becher, B. (2012). Rorγt+ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. J. Clin. Invest. *122*, 2252–2256.

Becher, B. & Pantelyushin, S. *Nature medicine* **18**, 1748–1750 (2012).

Wohn, C., Ober-Bloebaum, J.L., Haak, S., Pantelyushin, S., Cheong, C., Zahner, S.P., Onderwater, S., Kant, M., Weighardt, H., Holzmann, B., Reizis, B., Becher, B., Prens, E., and Clausen, B.E. Langerin^{neg} conventional dendritic cell-derived IL-23 drives imiquimod-induced psoriasis in mice. (manuscript submitted).

Kulig, P., Haak, S., Pantelyushin, S., and Becher, B., IL-12 axis in Aldara Psoriasis (Manuscript in preparation).

Conferences attended:

January 2013	NIF Winter School on Advanced Immunology Oral and poster presentations	Singapore, Singapore
May 2012	Fifth International Gamma-Delta T-Cell Conference. Oral presentation	Freiburg, Germany
April 2012	XXIX Meeting of the Swiss Immunology PhD students. Oral presentation	Wolfsberg, Switzerland
Octorber 2011	2nd International Conference on Immune Tolerance. Poster presentation	Amsterdam, The Netherlands